

POSITIVE REGULATORS OF *BACTEROIDES* CTNDOT
EXCISION AND TRANSFER

BY

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DISSERTATION

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Abstract

Tetracycline stimulates both excision and transfer of a *Bacteroides* Conjugative transposon CTnDOT. It was shown previously that a gene, *rteC*, is necessary for tetracycline-stimulated transcriptional regulation of the *xis2c* operon, which contains the excision genes. The protein encoded by this gene, RteC, did not have primary amino acid sequence homology to any known proteins in the databases. Accordingly, I sought structural homologs of RteC. A three-dimensional structure prediction by Robetta suggested that RteC might have two domains, and the C-terminal domain might have a winged helix motif. Based on the Robetta prediction, the human transcriptional factors E2F-4 and DP2 were identified as the most likely structural homologs of RteC. I made alanine substitutions within the putative DNA binding helix 3 region of RteC. Assays of *xis2c::uidA* activation by alanine mutants indicated that residues 174, 175, 178, 180, and 184 in helix 3 might contact the upstream region of P_E. The same alanine RteC mutants were used to test the effect of mutations on binding, and all mutants lost binding to P_E region. The upstream region of *xis2c* contained two inverted repeat half-sites. Mutational analysis of these half-sites showed that both half-sites are important for activity. In vivo GUS assays were

also done to test whether these half-sites are important for binding. Disrupted inverted repeat sequences resulted in loss of binding to P_E region. Thus, I have identified the DNA binding portion of RteC and DNA site to which it binds.

The tetracycline regulated excision operon, containing *xis2c*, *xis2d*, and *exc* of the *Bacteroides* conjugative transposon CTnDOT has been shown to mediate not only the excision of CTnDOT and but also the regulation of transcription of a large operon containing genes required for transfer of the CTnDOT transfer intermediate. In this study, the importance of two excision proteins (Xis2c and Xis2d) was examined to determine their roles in regulating the transcription level of the transfer operon. In-frame deletions in *xis2c* and *xis2d*, and deletion of *exc* reduced the in vivo transcription induction of the operon by 58%, 97%, and 16% respectively. Xis2c and Xis2d were shown to bind to the 450-bp DNA fragment immediately upstream of the first gene in the transfer operon but Exc did not exhibit any gel shift activity. However, Exc appeared to bind cooperatively when added to Xis2d and caused a super shift of the fragment. I obtained a footprint of Xis2d covering a 50-bp region immediately upstream of the promoter of the transfer operon. Mutations within this region had varying effects on the transcriptional activity in vivo and resulted in a loss of Xis2d binding. The same mutations also

affect a crude preparation of Xis2c in its ability to bind DNA. My results confirmed the importance of Xis2c, Xis2d, and Exc in the transcriptional regulation of the transfer genes.

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Chapter 1

INTRODUCTION

1.1 *Bacteroides* spp. and their clinical significance

Bacteroides are gram-negative obligate anaerobes. They form a major part of the normal bacterial flora of humans. *Bacteroides* spp. are predominant members of the human colon microbiota (41, 55). In the gut, they utilize complex polysaccharides that are not digestible by the host (55). In general, *Bacteroides* spp. are advantageous, providing nutrients for the host and stimulating mucosa cell turnover. However, they are also opportunistic pathogens. Colonic *Bacteroides* species such as *B. fragilis*, *B. thetaiotaomicron*, and *B. ovatus* can cause various infections, including blood stream infections and abscesses in the abdominal cavity, brain, or lung (6, 31, 49, 55). In the past, these anaerobic infections could be easily treated by antibiotics. But infections caused by *Bacteroides* spp. have become more difficult to treat because of increasing antibiotic resistance (11, 56, 57, 60, 69).

Human colonic bacteria, including *Bacteroides*, transfer resistance genes among themselves. They may also transfer or obtain such genes from bacteria that are passing through the colon (56). Proof that resistance gene transfer actually

occurs comes from the finding that DNA sequences of resistance genes from different species and genera of bacteria in the colon and in other sites are virtually identical (56). In particular, it was found that antibiotic resistance genes may be transferred between Gram-positive and Gram-negative bacteria (56).

Bacteroides in the human colon are resistant to β -lactam antibiotics (8, 83) and all aminoglycosides (12, 55). This is because *Bacteroides* produce β -lactamases (8, 46, 47, 62, 82, 84) and are unable to transport aminoglycosides (1). In years past, tetracycline was used to treat *Bacteroides* infections. However, tetracycline has become useless because nearly 100% (13) of all *Bacteroides* clinical isolates contain the tetracycline resistance gene, *tetQ* (42, 72, 73). A new tetracycline analog (tigecycline) seems to be effective but resistance has already begun to appear (N. Shoemaker, unpublished data). *Bacteroides* spp. also have become resistant to clindamycin, an antibiotic widely used to treat *Bacteroides* infections in the past (8, 83). The increase in resistance among *Bacteroides* spp. appears to be the result of acquisition of antibiotic resistance genes by horizontal gene transfer (11, 56, 57, 60, 69).

Hybridization analysis of community samples collected from three different time periods (pre-1970, 1970~1980, and 1996~1997) showed a dramatic

increase of resistance to tetracycline and erythromycin over time (69). Therefore, spread of antibiotic genes is not limited to clinical samples. All the tetracycline resistant strains contained *tetQ* (69), a gene that encodes a ribosome protection type of tetracycline resistance (43). Before 1970, 28% of community samples carried *tetQ* (6). Carriage was increased to 83% by 1996~1997 (69). Similarly, the number of strains containing erythromycin resistance genes has increased between the pre-1970 period and 1996 and 1997 (69). The majority of the erythromycin resistant strains carried either *ermF* or *ermG* (69). These two genes belong to the macrolide-lincosamide-streptogramin B (MLS) family of resistance genes (52, 94). These genes are also responsible for resistance to clindamycin, and this may explain increased resistance to clindamycin among *Bacteroides* spp.

Among *Bacteroides* spp., antibiotic resistance genes are acquired by horizontal gene transfer. Many *Bacteroides* strains contain one or more plasmids (13, 77, 90). Previous work showed that some antibiotic resistance genes were spread by horizontal transfer of these plasmids (29, 98). However, most of the antibiotic resistance transfer among *Bacteroides* spp. is now known to be mediated by conjugative transposons (CTns) (2, 18, 21, 33, 51, 61, 89). Most of the antibiotic resistance genes found in *Bacteroides* spp. are on mobile integrated elements such as

conjugative transposons (CTns) and non-replicating *Bacteroides* units (NBUs) (11, 57, 74, 97). CTns and NBUs are DNA segments that are normally integrated into the bacterial chromosome and can be excised and transferred from donor to recipient by conjugation (96).

1.2 The *Bacteroides* conjugative transposon, CTnDOT

Conjugative transposons (CTns) are DNA segments that are normally integrated in bacterial chromosome but can be excised and transferred from donor to recipient by conjugation. CTns are similar to transposons and temperate bacteriophages because they integrate into DNA (62). They resemble plasmids since the excised intermediate forms a covalently closed circle, and this circular intermediate contains a transfer origin (*oriT*) sequence that allows it to be transferred by conjugation (17, 38). CTns carry antibiotic resistant genes. Genes encoding resistance to tetracycline, erythromycin, kanamycin, chloramphenicol, streptomycin, sulfamethoxazole, and trimethoprim have been found on CTns (21, 89). Like self-transmissible plasmids, CTns have broad host ranges and are responsible for antibiotic resistance gene transfer among both gram-positive bacteria and gram-negative anaerobes (4, 17, 23, 44, 73).

The size of conjugative transposons ranges from 18 kb to more than 500 kb (64). CTns have genes required for excision, transfer, and conjugation. They also contain antibiotic resistance genes and other accessory genes. In *Bacteroides* species, smaller integrated elements such as NBU1, Tn5520, Tn4555 were found, and they have been called mobilizable elements (MTns). MTns are smaller than CTns, and they are caused by CTns to be excised and transferred from donor to recipient (59, 63). Co-resident plasmids that are not able to transfer by themselves are also mobilized by CTns. Sequence analysis of NBU1, Tn5520, and Tn4555 showed that these MTns, like the CTns, are member of lambda family of integrases (70, 87, 88). Conjugative and mobilizable transposons play an important role in antibiotic resistance transfer, but they are also able to transfer other metabolic traits (64).

The most studied conjugative transposons are the gram-positive conjugative transposon, Tn916, and the *Bacteroides* conjugative transposon, CTnDOT. Tn916 was first found in *Enterococcus faecalis* and it is the smallest known conjugative transposon with a size of 18 kb (67). It carries a tetracycline resistance gene, *tetM*, a gene that encodes a ribosome protection protein. CTnDOT is a conjugative transposon that was first found in a *Bacteroides* strain in a patient with a serious *Bacteroides* infection (64, 69). CTnDOT type elements play an important

role in transferring antibiotic resistance genes among *Bacteroides* species in the colon (62, 69, 97). The size of CTnDOT is 65 kb and it carries two resistance genes: a tetracycline resistance gene (*tetQ*), and an erythromycin resistance gene (*ermF*) (Figure 1.1). The *tetQ* gene encodes a cytoplasmic protein which protects the ribosome from tetracycline (20), while the *ermF* gene encodes a protein that modifies the 23S rRNA molecule, preventing erythromycin from binding to the ribosome (94). Both Tn916 and CTnDOT have similar series of steps in excision and transfer (64). When they are excised from the chromosome, a chromosomal 5-bp coupling sequence near the excision site is incorporated in the circular form (64) (Fig. 1.3). This coupling sequence is later introduced into the target site when the CTn integrates into recipient's chromosome (64). Tn916 and CTnDOT differ somewhat in the specificity of integration (64). Tn916 integrates into random sites in recipient's chromosome, while integration of CTnDOT is site-selective into about at least 7 sites on the *Bacteroides* chromosome (9, 64). Site selectivity of CTnDOT might occur because CTnDOT contains a 10-bp consensus sequence at the right end of the CTn, which has sequence identity to a 10-bp sequence next to the integration site (15). Transfer of both Tn916 and CTnDOT is stimulated by tetracycline (63). The mechanism of this stimulation, however, is different. In the presence of

tetracycline, CTnDOT transfer is regulated by three regulatory genes, *rteA*, *rteB*, and *rteC* (58, 76). Tn916 has no such regulatory genes.

Excision and transfer of CTnDOT occurs in three steps. At first, CTnDOT is excised from chromosome and forms a non-replicating double-stranded circle (Fig. 1.2). Once excised and circularized, this circle cannot replicate independently (64). Using primers that anneal to the ends of CTnDOT, a circular intermediate can be detected by PCR (15). If the CTn is in the chromosome, there are no PCR products because the 3' ends of primers are towards the chromosome (15). A PCR product is made only when the CTn is excised and circularized (15). Southern blot analysis has also been used to confirm the joined ends of the circular form (68).

When CTnDOT is excised from the chromosome, it makes staggered cuts 5-bp away from its ends (Fig. 1.3). The circular intermediate thus ends up having 5-bp from the donor chromosome. The sequences from the chromosome are called coupling sequences, but they are not complementary to each other (64). When the circle is formed, a small region of heterology is made (15, 80) (Fig. 1.3). The region of heterology is resolved either by replication following integrating or by repair enzymes (15, 57) (Fig. 1.3).

The non-replicating circular intermediate is nicked at an internal *oriT* site and a single strand is transferred from donor to recipient by conjugation (60, 64, 66). (Fig. 1.2) Mobilization genes (relaxase) are involved in the nicking of the DNA at the *oriT* site, and transfer genes form the mating bridge. The *oriT* site in CTnDOT has been narrowed down to less than 1kb.

Excision produces a double stranded DNA circle. (15, 64, 96) (Fig. 1.2). Integration requires super-coiled double stranded DNA. When the super-coiled DNA in *attDOT* was either cut or relaxed, integration was abolished (J. Laprise, unpublished data). Integration into the chromosome is done by a tyrosine recombinase type reaction (1, 15, 30, 50). Like phage lambda integration, integration of CTnDOT is non-homologous integration; sequence homology between the *att* site and the joined ends of the circular form is not required. Integration assays with a CTnDOT mini-element (a plasmid containing only the joined ends, the CTnDOT integrase, and the *oriT*) showed that disrupting the integrase abolished integration (15). This suggests that integrase is required for integration of CTnDOT (15). Integration in CTnDOT is not regulated by tetracycline (15).

1.3 Regulation of CTnDOT excision

CTnDOT excision and transfer are both regulated (Fig. 1.4). Excision is induced by tetracycline (15, 16, 64, 75), and is regulated by three regulatory proteins encoded by *rteA*, *rteB*, and *rteC* (62, 76). The *tetQ* (62) gene is the first gene in the operon followed by two genes, *rteA* and *rteB* (9). TetQ protects the ribosome from tetracycline (20). It has been shown that transcription of *tetQ*, *rteA*, and *rteB* is constitutive, whereas the translation of these genes is increased by tetracycline (92). Tetracycline stimulation is mediated by a translational attenuation mechanism, which involves the leader region of the *tetQ* operon (91, 92) (Fig. 1.5). Between the *tetQ* promoter and the *tetQ* start codon, there is an mRNA leader region containing hairpin structures (91, 92). The main hairpin structure has a stem composed of two sequences, Hp1 and Hp8 (91, 92). The ribosome binding site for *tetQ* is in Hp8 (91, 92). Within the leader region, there is 3-amino acid peptide that is required for regulation by tetracycline (91). Deletion analysis confirmed that between Hp1 and Hp8, another region, Hp2, is essential for tetracycline regulation of genes on the *tetQ* operon (91). Hp2 could form a hairpin structure with Hp1. In the presence of tetracycline, the Hp1-Hp2 structure is formed when ribosomes bound by tetracycline are stalled on the 3-amino acid peptide, making the *tetQ* ribosomal binding site

(RBS) available (91) (Fig. 1.5). This allows the ribosome to bind the RBS of *tetQ* and translate *tetQ* (91).

Previous studies identified both RteA and RteB as regulatory proteins based on amino acid similarity to other known two-component regulatory systems, with RteA being the histidine kinase-environmental sensor and RteB being the transcriptional activator (76). RteB activates the expression of a third gene, *rteC*, which controls the expression of genes essential for excision (40, 76). Previous work showed that RteC works as a positive regulator of excision genes since disrupted RteC abolished excision (16). When the *rteC* native promoter was replaced by the constitutive *tetQ* promoter, this construct was able to express both transcriptional and translational *xis2c::uidA* fusions in trans, suggesting that RteC itself, without RteA and RteB, is sufficient to express the excision gene operon (40).

In contrast to RteA and RteB, RteC did not have the primary amino acid sequence homology to known protein databases, and it did not have the part of obvious helix-turn-helix motif that many DNA binding proteins have. Also, the pI value of RteC is neutral, 6.9 (48). To check for possible structural motifs, I used the Helix-turn-helix prediction program NPS@ (http://npsa-pbil.ibcp.fr/NPSA/npsa_hth.html), and PSIPRED protein prediction server

(<http://www.psipred.net/psiform.html>). However, no significant motif was found.

Therefore, I decided to use a 3 dimensional structure prediction program, Robetta (35), in an attempt to find structural homologs of RteC. I was able to obtain a possible structure and function for RteC. I confirmed that RteC is a DNA binding protein that binds the upstream region of the excision operon to activate excision genes (48). In chapter 2, I describe my work on the characterization of RteC.

Previously, four genes critical for excision of CTnDOT (*intDOT*, *xis2c*, *xis2d*, and *exc*) were identified (16, 75). Originally, *xis2c* and *xis2d* were designated as *orf2c* and *orf2d*, but these genes are now re-named because they are clearly involved in excision.

The expression of the integrase gene, *intDOT*, is constitutive since integration occurs both in the presence and absence of tetracycline (15, 75, 88). The *intDOT* gene is at one end of CTnDOT (Fig. 1.1), and belongs to the lambda integrase family of tyrosine recombinases (16). In phage lambda, the cleavage and ligation reactions occur in the 7-bp overlap region including the integration site, and this lambda Int requires homologous sequences in this region (7, 93). CTnDOT Int is not as strict as lambda Int in its requirement for homology. Recombination occurs with both complementary and non-complementary coupling sequences (26). The

other three genes (*xis2c*, *xis2d*, and *exc*) are located downstream of *intDOT* (40) (Fig. 1.1). Excision of CTnDOT was abolished when these three genes were disrupted or deleted, while a deletion in the gene upstream of *exc*, *orf3* did not affect excision (16).

The excision of the well-studied integration elements typically requires two proteins, an integrase (Int) and an excisionase (Xis). Phage lambda requires integrase as well as a small basic protein called Xis to excise the integrated form (36). The integrase triggers the strand-exchange reaction (3). The excisionase controls the direction of the reaction. Xis triggers the excision reaction by bending DNA and enhancing co-operative binding of the integrase to the *att* site (45, 85, 99). The Gram-positive CTn, Tn916, also requires both an integrase and a *xis*-like gene for excision (53, 54). Xis proteins of phage lambda and Tn916 are small basic proteins that belong to the recombination directionality factors (RDF) family (37).

In CTnDOT excision, Xis2c and Xis2d might have similar properties to that of phage lambda Xis and other site-specific recombination systems. Xis2c is a 118 amino acid protein and its pI value is 10.7. Also it contains a helix-turn-helix (HTH) motif. Like Xis2c, Xis2d is a small basic protein (116 aa), with a pI value of

9.1 (80). Amino acid sequences of Xis2c and Xis2d do not have significant sequence homology to any proteins in the databases (80).

Exc is required for excision in vivo and it has topoisomerase function in vitro (16, 81). But excision still occurs in vivo at the wild-type level when the topoisomerase active site was disrupted (80). More recent studies have shown that Exc is stimulatory but not essential in vitro. This may be because Exc has a regulatory function in vitro, and Exc might stabilize the other excision components in vivo (80). The other explanation might be differences between in vivo and in vitro systems such as topology of DNA substrate (80). In the in vivo system, the substrates for excision, the left and right junction sequences of integrated CTnDOT (*attL* and *attR*), are located on the same DNA molecule (80). In the original in vitro system, these substrates were provided separately on plasmids, avoiding topological constraints (80). Exc may be necessary for excision from the substrate containing both *attL* and *attR* on the same molecule.

The Xer-mediated recombination system is an example of a recombination system that relies on topology of the substrates. Two recombinases, XerC and XerD, catalyze the recombination at the *psi* site, a site carried on pSC101 (5, 10, 22). The Xer-mediated recombination at this site requires the same oriented

recombination sites and is dependent on an intra-molecular structure (5). PepA and a phosphorylated ArcA (ArcA-P) are the two accessory proteins that are required for a specific synapse topology formation that facilitates the intramolecular recombination (19, 78). These proteins are involved in the Xer-mediated recombination. PepA forms the complex that is needed for excision, and regulates the transcription of the *carAB* operon (14). ArcA-P is involved in the excision complex formation (19, 78). Exc might function like PepA and ArcA-P to form a specific synapse topology for intramolecular recombination in vivo (80).

When CTnDOT is excised from the chromosome, the CTnDOT integrase creates staggered cuts near each end of the CTn (Fig. 1.3). These staggered cuts include 5-bp segments from chromosome, and these become the part of joined ends upon circularizing the excised CTn (15, 80). The 5-bp segments are called coupling sequences. They form a heteroduplex because the two 5-bp sequences are not complementary to each other (15, 80). The heteroduplex is resolved either by replication following integration or by repair enzymes (15, 57). The fact that both coupling sequences are found in the recipient with the equal frequency suggests that heterology is resolved in favor of either one coupling sequence or the other with nearly equal frequency, rather than resolving unidirectionally (63).

The two attachment sites at the ends of CTnDOT, *attL* and *attR*, have been localized using an in vitro competition assay (26). The minimal *attL* site is 153-bp and this sequence is composed of CTnDOT sequence (138 bp) and bacterial chromosome sequence (15 bp) (26). The minimal *attR* site is 179-bp and contains 164-bp of CTnDOT sequence and 15-bp of bacterial chromosome sequence (26). In both cases, the 15-bp bacterial chromosome sequences include the 5-bp coupling sequence. Mutational analysis and gel shift assays showed that a 6-bp region in *attR* is required for both excision and IntDOT binding (26). Using insertional mutagenesis, regions in *attL* site that are essential for excision were identified (26). The mutations might interfere with protein-protein or protein-DNA interactions. Similar mutations in the *attR* site did not affect excision, suggesting that excision of CTnDOT is asymmetrical regarding protein binding sites, and also involves multiple protein-DNA interactions (26).

Exposure to tetracycline is important since there is no excision or transfer in the absence of tetracycline (76, 92). The operon which contains the tetracycline resistance gene, *tetQ*, and two other regulatory genes, *rteA*, and *rteB* is critical for both excision and transfer of CTnDOT (9) (Fig. 1.5). It has been demonstrated that RteC acts as a positive regulator of the CTnDOT excision genes

in the *xis2c* operon (40). Thus RteC is important because it directly regulates the excision of the CTnDOT.

1.4 Regulation of CTnDOT transfer

Like excision, transfer of CTnDOT is also regulated by tetracycline (Fig. 1.5). The size of the CTnDOT transfer region is 18kb and it contains factors that mediate transfer; the transfer origin (*oriT*), mobilization genes (*mob* genes), and transfer genes (*tra* genes) (Fig. 1.1). The transfer region is located downstream of *rteC*, and this region is sufficient for CTnDOT transfer. That is, if this 18-kb transfer region is cloned into a non-mobilizable plasmid like pLYL05 (L.Y. Li, unpublished), the new plasmid, pLYL72 (38), becomes self-transmissible. Surprisingly, transfer of pLYL72 was not regulated by tetracycline.

It has now been shown that the excision operon regulates the transfer of CTnDOT. When pKSO1 (16), the plasmid containing the excision operon, was placed in trans with pLYL72 (38), transfer of pLYL72 plasmid became regulated by tetracycline. In the absence of pKSO1, transfer of pLYL72 was constitutive and tetracycline did not have any effect on transfer of this plasmid. Therefore, it appears that excision genes are involved in regulation of both excision and transfer of

CTnDOT. By coordinating regulation of excision and transfer, intact CTnDOT should be fully excised before transfer, insuring integration of the entire CTnDOT into recipient's chromosome.

Sequence analysis of the 18-kb transfer region showed that the putative *tra* genes (designated *traA* to *traQ*) are transcribed in the same direction (11). To test whether the *tra* genes are part of a single operon, RT-PCR was performed (34). Primers were designed to amplify the junction between each two genes. All reactions made RT-PCR products, suggesting that these genes are transcribed as part of single mRNA (34). An insertional mutation between *traA* and *traB* caused a polar effect on downstream genes, including *traG* (11), and the transfer of CTnDOT was abolished (34). RT-PCR analysis of *tra* genes in the presence and absence of tetracycline showed that *tra* genes are regulated at the transcriptional level (34).

The central regulatory proteins, RteA, RteB, and RteC are involved in expression of the excision gene operon (40). To test the effects of RteC and the excision proteins on *tra* gene expression, the following system was constructed. The integrase gene and the joined ends of NBU1 or NBU2 were used to insert the central regulatory genes, *tetQ*, *rteA*, and *rteB* (Ω QAB) or *tetQ*, *rteA*, *rteB*, and *rteC* (Ω QABC) into the chromosome (97). The NBU integrase gene and end sequence

were used because the inserted DNA cannot be excised from the chromosome; the excision genes of NBU1 or NBU2 have been removed (71). Both the excision region (pKSO1) and the transfer region (pLYL72) were provided on plasmids, and RT-PCR was used to measure expression of *tra* operon (34). When both the central regulatory genes (Ω QABC) and the excision region (pKSO1) were present, expression of the *tra* operon was up-regulated by tetracycline (34). Without pKSO1, *tra* operon expression was low and constitutive. Previous work showed that pLYL72 was transferred constitutively without the central regulatory genes (38). Therefore, both the excision region and the central regulatory region are required for up-regulation of *tra* gene expression (34).

RteC regulates excision gene expression. To test whether RteC also controls the *tra* operon directly, the excision operon was placed under control of a heterologous promoter, P_{susA} (34). P_{susA} is a maltose-regulator promoter, and by placing the excision operon under control of this promoter, expression of excision genes were rendered independent of tetracycline (24). The new construct containing the excision region was called pGRW3. The expression level of the excision genes were measured by in vivo GUS assays, using a translational *traA::uidA* fusion pGW40.5 (34). The fusion was used as an indicator rather than measuring pLYL72

transfer frequency because of potential toxicity caused by overproduction of transfer proteins (34). GUS activity was up-regulated in the presence of pGRW3 (34). When excision of CTnERL, a CTn that is exactly the same as CTnDOT except that it does not have an *ermF* gene, was tested in the presence of pGRW3, CTnERL was excised, and excision was independent of tetracycline (34). The transfer genes were also expressed independent of tetracycline (34). Therefore, the excision region is sufficient to regulate *tra* operon expression (34).

It is interesting to note that Xis2c, Xis2d, and Exc are involved in both excision and transfer regulation of CTnDOT (Fig. 1.5). Both conjugative transfer of pLYL72 and the expression of transfer proteins were enhanced by tetracycline when the excision proteins (Xis2c, Xis2d, and Exc) were present (96). Xis2c and Xis2d did not have sequence homology to proteins in known protein databases, but they are small basic proteins. Xis2d contains a helix-turn-helix motif that many DNA binding proteins have. Therefore, these two proteins might be DNA binding proteins. Preliminary gel shift analysis showed that both Xis2c and Xis2d bind the upstream region of *traA*. Another excision protein, Exc did not bind this region in vitro. It has been shown that many topoisomerases alter DNA topology to modulate gene expression (27, 28). Exc might have the same function and modify local

supercoiling to enhance conjugative transfer of CTn and expression of transfer genes.

Currently, I am working on identifying Xis2c and Xis2d binding sites within the upstream region of *traA*. Both in vivo GUS assays and in vitro footprint assays will be done to determine the binding sites of Xis2c and Xis2d (Chapter 3).

Previously, it was shown that pKS05, a plasmid containing a region of 3' end of the *exc* gene, inhibits pLYL72 transfer (96) (Fig. 1.5). This 500-bp region also includes a small open reading frame (ORF) that was designated as *rteR*. To see if this ORF encodes protein, two stop codons were introduced into the putative ATG start codon and the second codon (34). The mutated *rteR* still inhibited the transfer, suggesting that the ORF does not encode RteR protein but may encode a regulatory RNA (34). To determine whether *rteR* was being transcribed and whether its expression was regulated by tetracycline, Northern blot analysis was done to compare RteR levels in BT4007 cells which carry CTnDOT, in the presence and absence of tetracycline (34). RteR was little less than 100nt in size and it was expressed constitutively (34). The 5' end of *rteR* has now been shown to be located at the end of *exc* and the 3' end has been located.

Some small RNAs bind to mRNA and attract protein Hfq, causing degradation of RNA (25, 32, 39, 79). To test if RteR makes *tra* mRNA unstable,

rifampin was added to the medium and RNA was prepared from cells. Rifampin binds the beta-subunit of RNA polymerase and inhibits DNA-dependent RNA polymerase, resulting in prevention of transcription of RNA. RT-PCR was used to detect the amount of *traG* message, and *traG* mRNA was not destabilized in the presence of RteR (34). Also, Hfq gene homolog has not been found in the *B. thetaiotaomicron* genome sequence (34). Therefore, RteR may not destabilize mRNA to down-regulate transfer at the transcriptional level. Instead, RteR might cause early termination by binding to mRNA, which was seen in another mobile element, pAD1 (86). Alternatively, RteR might destabilize *tra* genes other than *traG*.

1.5 Concluding remarks

In my dissertation, I focus on positive regulators of CTnDOT excision and transfer. Excision and transfer of CTnDOT are complex processes and are tightly regulated. At first, I characterized RteC, a positive regulator of the excision gene operon. This dissertation describes how I used the Robetta protein structure prediction server to test my hypothesis that RteC is a DNA binding protein that is involved in transcriptional activation. The excision genes play an important role in enhancement of both excision and transfer. As a second stage of my project, I

decided to follow up an observation by a previous student. I confirmed that two excision proteins, Xis2c and Xis2d, are DNA binding proteins that bind upstream region of transfer operon and regulate the expression of transfer genes. And Exc interacts cooperatively with Xis2d to enhance binding of Xis2d to the upstream region of *traA* in vitro.

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Figures

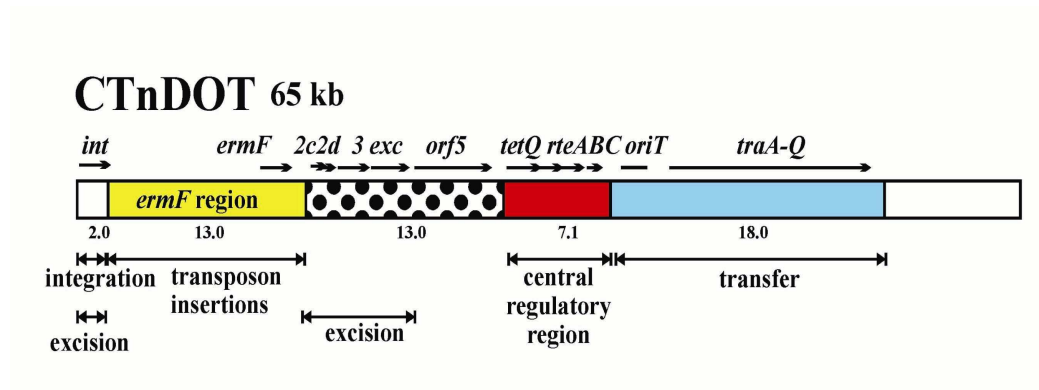


Figure 1.1 Map of CTnDOT

CTnDOT is 65kb, and it carries genes that confer resistance to erythromycin (*ermF*) and tetracycline (*tetQ*). At one end of CTn, integrase gene, *int* or *intDOT*, is located. The excision operon (*xis2c-2d-exc*) is 13kb and is required for both excision and regulation of transfer. The 7.1 kb central regulatory region contains *tetQ*, *rteA*, *rteB*, and *rteC*. The transfer region is required for transfer and is composed of an *oriT* site, *mob* and *tra* genes. The size of the transfer region is 18kb. Figure 1.1 is from Nadja Shoemaker.

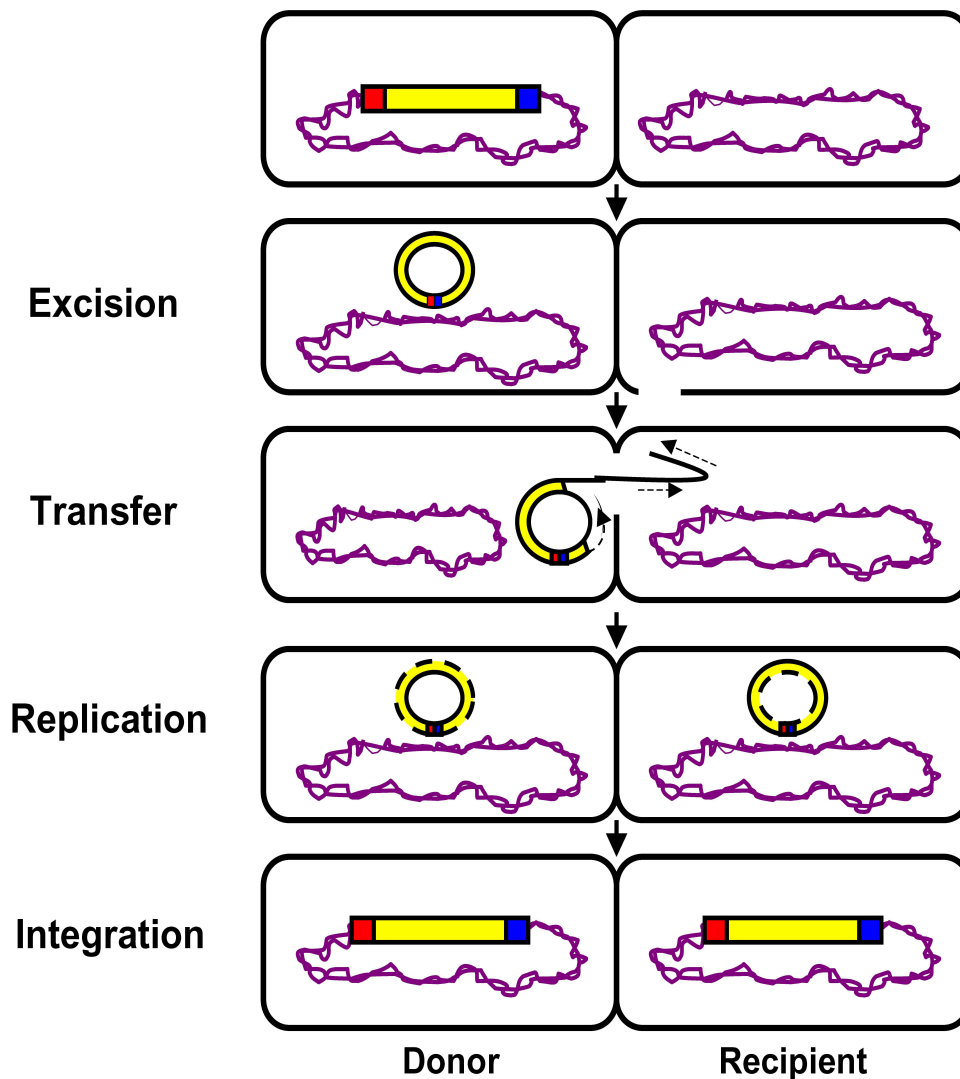


Figure 1.2 Model for the steps in CTnDOT excision and transfer

The integrated conjugative transposon (rectangle) in the chromosome of donor excises and forms a non-replicating circular intermediate. A single-stranded nick at the origin of transfer (*oriT*) in the circular intermediate occurs, and the nicked strand is transferred from the donor to recipient. In the recipient, the complementary strand is synthesized and the double-stranded intermediate integrates into the chromosome. The copy remaining in the donor is believed to reintegrate (probably in a different site) into the donor chromosome (97).

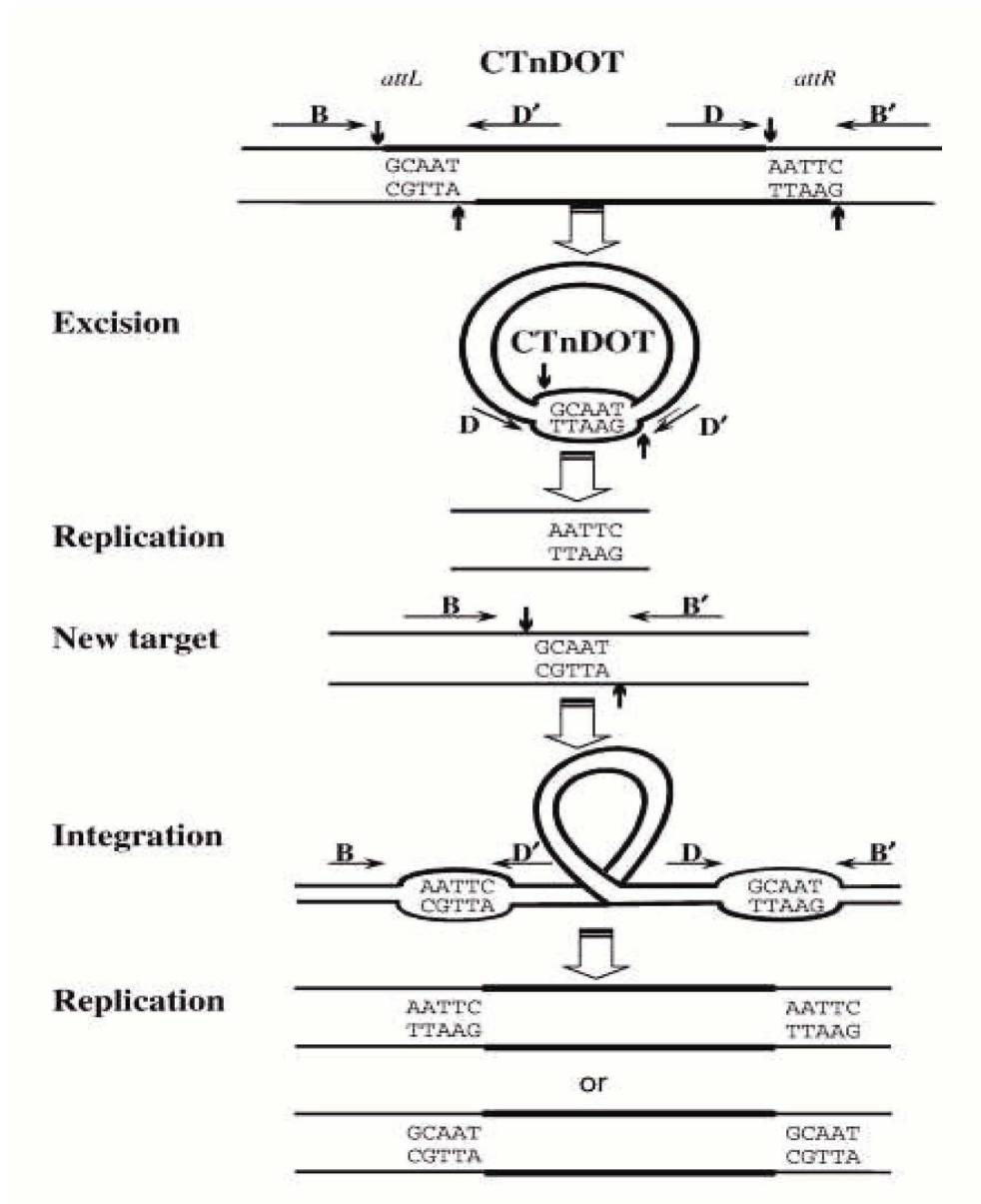


Figure 1.3 A schematic representation of excision and integration reactions of CTnDOT Int (IntDOT)

5-bp staggered cuts adjacent to the 5-bp coupling sequences (indicated by bold arrows) are made by IntDOT. The circular intermediate is created with a region of heterology. During transfer, the recipient receives one of the two coupling sequences. In the recipient's chromosome, a new target site is selected and the element integrates using the staggered cuts. There are conserved inverted repeat sequence in both *attL* and *attR*. These flank the coupling sequence, B and D' on *attL*, and D and B' on *attR*. Figure 1.3 is an adaptation of Dichiara et al (26).

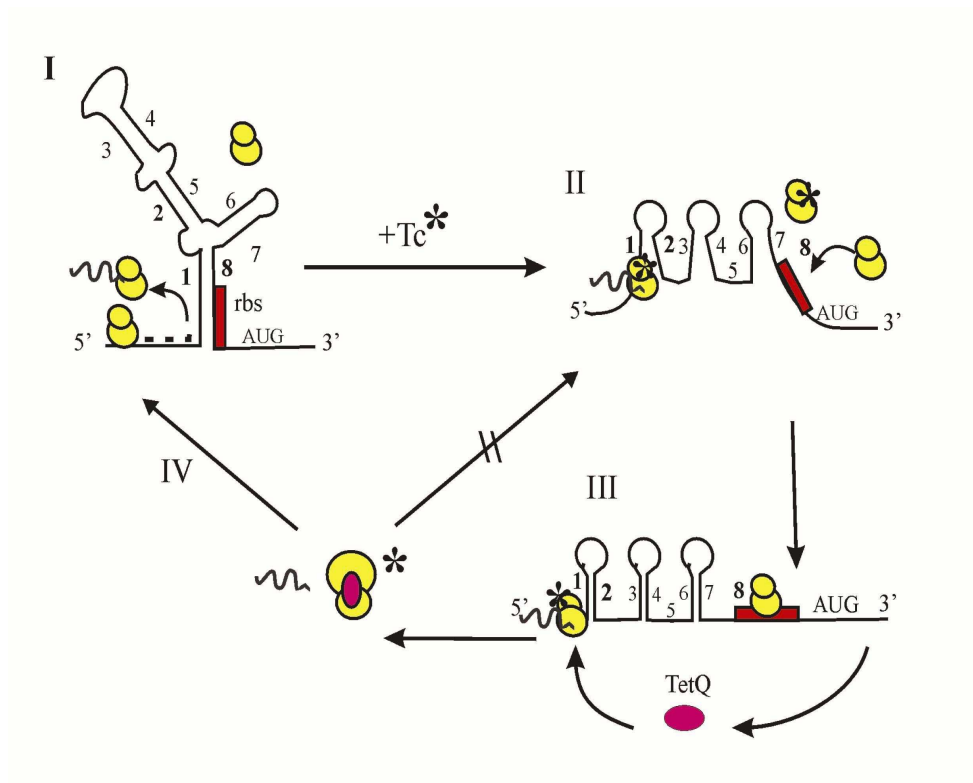


Figure 1.5 Proposed model for translational attenuation by tetracycline

In the absence of tetracycline (step I), mRNA is bound by ribosomes and the leader tripeptide are translated (dashed line). The ribosome binding site (rbs) of *tetQ* is in Hp8, and is not accessible because of the Hp1-Hp8 hairpin. In the presence of tetracycline (step II), ribosomes bound by tetracycline (indicated by an asterisk) stall on the leader peptide mRNA, altering mRNA structure. The ribosome binding site becomes available, and the *tetQ* gene is translated by ribosomes that are not bound by tetracycline. The bound tetracycline is released from the ribosome because TetQ alters ribosome conformation (step III). The altered ribosome returns to step I (step IV). Since ribosome is not bound by tetracycline, the mRNA structures which embed the ribosome binding site can be reformed. Figure 1.5. is an adaptation of Wang et al (91).

Chapter 2

CHARACTERIZATION OF *BACTEROIDES* CTNDOT REGULATORY PROTEIN, RTEC

Figures 2.1~2.6 and Table 2.1 have been published as part of the manuscript entitled “Characterization of the *Bacteroides* CTnDOT regulatory protein, RteC”, by Park and Salyers, J.Bacteriol., 2011.

2.1 Introduction

Bacteroides spp. are one of the numerically predominant groups of bacteria in the human colon, where they account for about 30% of the microbiota (15). *Bacteroides* spp. can cause serious opportunistic infections if they escape from the colon due to abdominal surgery or other trauma. Infections caused by *Bacteroides* spp. are difficult to treat because of increasing antibiotic resistance. Antibiotic resistance genes are being spread among *Bacteroides* spp. by horizontal gene transfer (16, 18, 19). CTnDOT is a conjugative transposon that was found originally in a *Bacteroides* strain that was isolated from a patient with a serious *Bacteroides* infection (16, 22). Subsequent studies showed that conjugative transposons such as CTnDOT type elements play an important role in transferring

antibiotic resistance genes among *Bacteroides* spp. in the colon (22). The first step in CTnDOT transfer is excision from the chromosome to form a non-replicating circular intermediate (28). Previously, an operon containing genes important for excision was identified (*xis2c*, *xis2d*, and *exc*) (4). These genes are regulated at the transcriptional level (14). Excision is induced by tetracycline (3, 4, 20, 23). Regulation appeared to be mediated by three regulatory proteins encoded by *rteA*, *rteB*, and *rteC* (5). The *tetQ* gene is the first gene in the operon that contains *rteA* and *rteB* (9). Transcription of *tetQ*, *rteA*, and *rteB* is constitutive, whereas the translation of these genes is increased during exposure to tetracycline (27). Increased protein production is due to a translational attenuation mechanism (26, 27). Both RteA and RteB are clearly regulatory proteins based on amino acid similarity to known two-component regulatory systems, with RteA being a histidine kinase-environmental sensor and RteB being a transcriptional activator (24). RteB activates the expression of *rteC*, which in turn controls the expression of genes essential for excision (14, 24). Previously, it was demonstrated that disruption of *rteC* abolished excision, suggesting that RteC acts as a positive regulator of excision genes (4). When *rteC* was placed under control of the *tetQ* promoter, so that *rteA* and *rteB* were no longer needed, and introduced in trans with both the transcriptional and

translational *xis2c-uidA* fusions, the construct stimulated the expression of the fusions (14). Therefore, RteC itself is sufficient to express the excision gene operon (14, 24). My hypothesis was that RteC is a DNA binding protein that binds upstream of the promoter of the *xis2c* operon, P_E. Yet RteC did not have primary amino acid sequence homology to any known proteins in the databases. Accordingly, I decided to search for structural homologs of RteC to obtain insights into the possible structure and function of RteC.

2.2 Materials and methods

Strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1.

The concentrations of antibiotics used were: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 15 µg/ml; kanamycin (Kn), 50 µg/ml; ceftiofur (Cef), 20 µg/ml; erythromycin (Em), 10 µg/ml; gentamicin (Gen), 200 µg/ml; rifampicin (Rif), 10 µg/ml. All the *E.coli* strains were grown in Lysogeny Broth (LB) or on LB agar at 37°C and the *Bacteroides* strains were grown in either Trypticase Yeast Extract Glucose (TYG) medium or Supplemented Brain Heart Infusion (BHIS) medium at 37°C under anaerobic conditions.

Site-directed mutagenesis

Site-directed mutagenesis was done using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and iProof High-Fidelity DNA Polymerase (Bio-Rad). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). Plasmid pGFK24.1 (K. Moon, unpublished), a pCR2.1 vector (Invitrogen), containing the 300-bp region upstream of the *xis2c* operon, was used as a DNA template in site-directed mutagenesis of the upstream region of the excision operon promoter (P_E). Plasmid pGFK70.1 (K. Moon, unpublished), a pCR2.1 vector (Invitrogen), containing His₆-tagged *rteC*, was used as a DNA template for site-directed mutagenesis of RteC_{His6} for in vivo GUS assays. The PCR amplicons containing different mutations were transformed into *E. coli* DH5αMCR competent cells (Bethesda Research Laboratories Inc., Gaithersburg, MD), and cells were grown in Lysogeny Broth (LB) agar medium containing ampicillin (100 µg/ml) or kanamycin (50 µg/ml) overnight. Plasmids were isolated, and then sequenced to confirm the mutation (UIUC Core DNA Sequencing Facility, Urbana, IL). For site-directed mutagenesis of the upstream region of P_E, the *SphI-SmaI* restriction fragments were isolated and ligated into the *SphI-SmaI* site of pMJF2 (6) to create an *xis2c::uidA* translational fusion, and then the *SphI-PvuII* fragments containing the

xis2c::uidA fusion were cloned into the SphI-NruI site of an *E.coli-Bacteroides* shuttle vector, pC-COW (9). For site-directed mutagenesis of RteC_{His6}, the *NcoI-SmaI* restriction fragments were isolated and ligated into the *NcoI-SmaI* site of pJPARK7 to replace wild-type RteC_{His6} with RteC_{His6} mutants. pJPARK7 is a pAFD1 vector containing pP_Q-*rteC*_{His6}.

Triparental matings

The plasmids containing wild type RteC, mutant forms of RteC, or mutant forms of P_E were mobilized into *Bacteroides* strain BT4001 via triparental matings. In each case, the two donors were *E.coli* DH5 α MCR, which contained either mutations in the P_E region or RteC mutants, and *E.coli* HB101, which contained the IncP α plasmid RP1 (21). RP1 cannot replicate in *Bacteroides* spp., but it does mobilize *E.coli-Bacteroides* vectors from *E.coli* donors to *Bacteroides* recipients (21). Matings were done on nitrocellulose filters. The plasmids were then mobilized into BT4001, a *Bacteroides* strain which contains pJPARK7, a pAFD1 vector containing pP_Q-*rteC*, or BT4001, which contains pGFK43.11, a pC-COW vector containing the P_E *xis2c:: uidA* fusion.

GUS assay

The *uidA* reporter gene on pMJF2 encodes an *E.coli* β -glucuronidase (GUS). GUS assays were done by the procedure of Feldhaus et al (6). One unit was defined as 0.01A₄₁₅U per min at 37°C. Protein concentrations were determined by the method of Lowry et al (12).

Overexpression of RteC

A promoterless *rteC* gene was amplified from BT4007 using PCR. High-fidelity PCR amplification was done by PfuUltra High Fidelity DNA Polymerase (Stratagene). The 670-bp *rteC* PCR product was cloned into pCR-Blunt (Invitrogen). An *NdeI-XhoI* fragment was cloned into the *NdeI-XhoI* sites of pET28b (Novagen), creating His₆-tagged *rteC*. *Escherichia coli* BL21 CodonPlus (DE3) RIL (Stratagene) was used as the host strain for wild-type RteC. Cells were grown overnight at 37°C in 10ml Luria Broth (LB) medium with chloramphenicol (50 µg/ml) and kanamycin (30 µg/ml). The overnight cultures were used to inoculate 500 ml of LB medium containing chloramphenicol (50 µg/ml) and kanamycin (30 µg/ml). The culture was incubated with vigorous shaking at 37°C. Cells were grown until O.D.₆₀₀ was 0.3~0.4, and isopropyl-D-thiogalactopyranoside (IPTG) was added

to a final concentration of 1mM. Cells were grown at 25°C for 3 to 4 hours with vigorous shaking. Cells were harvested by centrifugation at 7,000 rpm for 20 min at 4°C. The supernatant fluid was discarded, and the cells were stored at –80°C until use. Overexpression of His₆-tagged RteC protein was confirmed by Western blotting with Mouse anti-His Antibody (GE Healthcare) as a primary antibody and anti-mouse IgG HRP (Promega) as a secondary antibody. The tagged RteC was tested in *Bacteroides* strain 4001 containing the P_E *xis2c::uidA* fusion to ascertain that it activated the *xis2c* operon similarly to wild type RteC.

Cell extract preparation and purification of RteC

The frozen cell pellet was resuspended in 10 ml lysis buffer (50 mM Tris; pH8.0, 1 M NaCl) to which one Complete Mini EDTA-free protease inhibitor tablet (Roche Applied Science) had been added. The cell suspension was frozen on dry ice and thawed in 37°C water bath 3 times. Then the cells were lysed by sonication (15 sec sonication and 2 min on ice, 6 times). Cell lysate was separated by centrifugation at 7,000 rpm for 20 min at 4°C. The supernatant was incubated with 1ml cobalt resin (Pierce) that had been equilibrated with lysis buffer at 4°C for 1 hour, and passed over a gravity-flow column. The column was eluted with elution

buffer (50 mM Tris-HCl ; pH 8.0, 1 M NaCl, and 150 mM imidazole). The crude cell extract or the partially purified RteC_{His6} was dialyzed overnight in a Slide-A-Lyze cassette (Thermo Scientific) against dialysis buffer (50 mM Tris-HCl; pH 8.0, 1 M NaCl, 1 mM DTT, 1 mM EDTA, 20% glycerol), and stored at -80°C until use. The partially purified RteC_{His6} was analyzed by both SDS-PAGE and native gel and subjected to Western blot to determine the molecular weight.

Gel shift assays

The 260-bp DNA fragments utilized in the gel shift assay experiments were prepared from plasmids containing wild-type or mutated P_E region by restriction enzyme digest (*SphI* and *SmaI*) followed by gel extraction. DNA fragments were labeled with gamma-[³²P] ATP using PCR, and purified. The DNA fragment (approximately 5ng) and either partially purified RteC or RteC cell extract were mixed in 10μl binding buffer and were incubated for 15 min at room temperature. The 5X binding buffer contained 250 mM Tris-HCl (pH 8.0), 5 mM EDTA, 250 mM NaCl, 50% glycerol, and 3.6 μg/ml herring sperm DNA. The binding mixture was loaded onto a 6% DNA Retardation Gel (Invitrogen), and 0.5X

Tris-borate-EDTA buffer (pH 8.0) was used as the running buffer. After electrophoresis, the gels were dried and subjected to autoradiography.

2.3 Results

Structural homologs of RteC

The linear RteC amino acid sequence did not have significant homology to any proteins in the databases (14). Moreover, unlike many DNA binding proteins, which have a high pI, the predicted pI of RteC was 6.9. Accordingly, I decided to look for the structural homologs instead of relying on the linear amino acid sequence. To find the possible structural homologs of RteC, I used the protein structure prediction server Robetta (10). The Robetta server uses methods including PSI-BLAST (1) and the Rosetta fragment-insertion to generate a hypothetical structure (5, 10). Models can be built by the de novo Rosetta prediction method (1, 5, 10). Based on the Robetta (10) prediction, I found that RteC might have two domains, including a C-terminal domain that contains a winged helix motif (8). A winged helix motif is a subfamily of the helix-turn-helix motif (8). This motif consists of three alpha-helices and two beta strands (8). Two anti-parallel beta strands make wings, and these wings flank the third helix (8). Typically, the third helix works as a

recognition helix and interacts with the major groove of DNA (8). Based on the Robetta structural prediction, when compared to structures in the database, human transcriptional factors E2F-4 (29) and DP2 (29) were the closest homologs with Z-score of 6.46. *E.coli* LexA repressor (7), and *E.coli* arginine regulator ArgR (11) could also be structural homologs of RteC. The fact that all of these homologs are known DNA binding proteins supported the hypothesis that RteC is a DNA binding protein. E2F-4 forms a heterodimer with the other transcriptional factor, DP2 to efficiently bind DNA, and these two proteins share sequence homology in their DNA binding domains (29). I used ClustalW (25) to align the putative C-terminal domain of RteC, amino acid residues 111 to 217, with DNA binding domains of the human transcriptional factors, E2F-4 (29) and DP2 (29), and the secondary structure of the putative RteC DNA binding domain was obtained (Fig. 2.1). Since helix 3 works as a recognition helix in many winged helix proteins, the putative helix 3 region in RteC (residues 174 to 189) was subjected to mutation.

Alanine substitutions in the putative RteC helix 3

I made 7 individual alanine substitutions within the putative helix 3 region in RteC. Alanine substitutions were constructed at K174, D175, R178, F179,

Y180, K184, and E189 (Fig. 2.2A in bold). The RteC alanine mutants were then tested for the ability to activate the wild-type *xis2c::uidA* fusion. I found 3 amino acid residues that were fully conserved in RteC (residues 174, 178, and 180), E2F-4 (residues 118, 123, and 125) and DP2 (residues 55, 57, and 59). The single alanine substitution of these amino acids resulted in decreased activity to the background level (Fig. 2.2B). Mutations at D175 and K184 rendered defective for transcription activation of *xis2c::uidA* (Fig. 2.2B). Mutations at F179 showed 20% decreased activity, while an alanine substitution at E189 did not affect the activity compared to the wild-type (Fig. 2.2B). These results show that portions of helix 3 of RteC is important for RteC activity.

Site-directed mutagenesis of upstream region of P_E

I also wanted to define the RteC DNA binding site upstream of P_E. Preliminary analysis had suggested that the 20-bp between –51 and –70 relative to the *xis2c* transcriptional start site might be important for activity (14). To identify the important base pairs within this 20-bp region, 5 different 4-bp mutations were made within this region (Fig. 2.3A). The changes were complementary to conserve the melting temperature characteristics. The effects of mutations on activity were

measured by an in vivo GUS assay. The results of each set of 4-bp mutations within the 20-bp region upstream of P_E on the *xis2c::uidA* are shown in Figure 2.5B. Cells containing both wild-type 20-bp and wild-type RteC exhibited a 100-fold increase in GUS activity compared to cells without RteC (Fig.2.3B). The 4-bp change between –67 and –70 did not affect the GUS activity, while the 4-bp mutations of the downstream 16-bp resulted in more than 2-fold decreased GUS activity (Fig. 2.3B). When the entire 20-bp region was changed, the activity was decreased 8-fold (14). The 20-bp region contained a 7-bp inverted repeat half-site. Another inverted repeat half-site was located immediately downstream of the 20-bp sequence, from –49 to –43 relative to the *xis2c* transcriptional start site. The fact that mutations in the part of 20-bp that contained one inverted repeat half-site resulted in 8-fold decreased activity (3), but did not decrease GUS activity to the background level may mean that both half-sites must be mutated to eliminate gene expression.

More site-directed mutagenesis was done to determine important base pairs adjacent to the 16-bp region from –51 to –66. To test our hypothesis that RteC may bind the two inverted repeat half-sites and that the -33 region may be important for activity because it is part of the P_E promoter, 4 different sets of mutations were made within the upstream region of P_E. One inverted repeat half-site mutation from

–43 to –49, the other inverted repeat half-site mutation from –53~–57, and the –33 region were changed by site-directed mutagenesis (Fig.2.4A). The region from –35 to –38 was called the –33 region, because of its sequence similarity with other *Bacteroides* promoter regions (2). In *Bacteroides*, there are conserved –7 and –33 regions which are analogous to the –10 and –35 region in *E.coli* (2). The 20-bp region from –45 to –65 relative to the *xis2c* transcriptional start site was chosen since mutation of 4-bp from –67 to –70 did not affect the activity (Fig.2.4A). Changes in the two inverted repeat half-site mutations were transition mutations to destroy the inverted repeat sequence. The 4-bp mutation from –35 to –38, the –33 region, and the mutations from –45 to –65 were to complementary sequences to conserve the melting temperature characteristics. When the one inverted repeat half-site from –43 to –49 (TCACGTA) was mutated, the activity was decreased about 2-fold compared to the wild-type control (Fig.2.4B). The other inverted repeat half site mutation from –53 to –57 (TACGT) showed about 12-fold decreased activity (Fig.2.4B). With the mutated –45 to –65 region, the activity was decreased almost to the background level (Fig.2.4B). The activity was decreased about 10-fold in the case of the –33 region mutation (Fig.2.4B). This result suggested that both inverted repeat half-sites are important for RteC binding.

The effect of P_E region mutations on binding of RteC to DNA

My results supported the hypothesis that RteC is a DNA binding protein that binds the upstream region of P_E and activates the expression of excision genes. To confirm that RteC binds DNA and recognizes the inverted repeat sequence within upstream region of P_E, gel shift assays were done. Partially purified empty vector (no RteC) was used as a negative control (Figure 2.5B, lanes 2, 5, 8, and 11). In the gel shift assays, wild-type RteC binding to wild-type P_E resulted in a super shift (Figure 2.5B, lane 3). However, when either inverted repeat half-site or the entire inverted repeat sequence was disrupted, the super shift no longer occurred (Figure 2.5B, lanes 6, 9, and 12). This result showed that RteC can bind DNA in the P_E region and confirm that both inverted repeat half-sites are important for RteC binding.

2.4 Discussion

RteC does not have primary amino acid sequence homology to any known proteins in the databases. To get past this, I used the protein structure prediction program, Robetta (10) to predict the structure of RteC in the hopes of finding other proteins with structural homology. I chose Robetta to predict the

structure of RteC because it can generate a model for an entire sequence without sequence homology to proteins of known structure (5, 10). Previously, I used the PSIPRED protein structure prediction server (13). The secondary structure prediction showed that RteC might contain many α -coils and β -sheets, but no clear motifs stood out. Thus the Robetta (10) program has been the most informative. Based on the Robetta prediction, I found 4 structural homologs of RteC and all were involved in transcriptional regulation. Those homologs were E2F-4 (29) and DP2 (29), *E.coli* LexA repressor (7), and *E.coli* arginine regulator ArgR (11). These proteins all contain winged helix motifs in their DNA binding domains. Guided by this, I could see that RteC also appears to have such a motif. The pI of RteC was predicted to be 6.9, and the putative structural homologs of RteC, human transcriptional factor E2F-4 (29) and DP2 (29), and *E.coli* LexA (7) also exhibited neutral pI values. However, DNA binding domains of these proteins have higher pI values. In RteC, the predicted pI of the C-terminal domain is 9.1. This suggested that RteC amino acid residue 111~217 might be involved in DNA binding. All 4 structural homologs of RteC work as dimers, and electrophoretic analysis under native condition confirmed that RteC forms a tetramer (Fig. 2.6B, lane 2). Since E2F-4 (29) and DP2 (29) were the closest structural homologs of RteC, based on the

Robetta prediction, these two transcription factors were chosen for comparison to RteC. The putative secondary structures of RteC DNA binding domains were obtained by aligning DNA binding domains of E2F-4 and DP2 with the C-terminal domain of RteC. Since the helix 3 works as a recognition helix in most winged helix proteins (8), the putative helix 3 of RteC was subjected to alanine substitutions. Western blot analysis confirmed that RteC mutants were stable and expressed at the same level as the wild –type.

Based on sequence alignment by ClustalW (8), three amino acid residues in the putative helix 3, K174, R178, and Y180, are fully conserved amongst RteC, E2F-4, and DP2. Both E2F-4 (29) and DP2 (29) use a conserved Arg-Arg-XXX-Tyr-Asp motif to contact half of the palindromic sequence CGCGCG. (29). In E2F-4 and DP2, three amino acid residues in an RRXYD motif, two arginines and a tyrosine, directly contact the DNA (29), and both R178 and Y180 in the putative RteC helix 3 were aligned with two of these three residues that are responsible for DNA contact. The alanine mutation at D175 resulted in about 10-fold decreased activity. In E2F-4 and DP2, the aspartate in RRXYD motif makes charged hydrogen bonds to both arginines and appears to stabilize this arrangement (29). Both aspartates in the RteC putative helix3 might stabilize the other amino acids that contact DNA bases, and

this might explain the more than 10-fold decreased activity. Taken together, my results support the hypothesis that RteC is a winged helix type DNA binding protein and that the predicted DNA binding region, helix 3, is important for binding.

Preliminary evidence suggested that RteC might bind somewhere in the 20-bp upstream of P_E (-51 to -70). To narrow down the region that is important for activity, we made 5 sets of 4-bp mutations within this 20-bp region, and found the mutation of the first 4-bp (-67 to -70) did not have any effect (Fig. 2.3B). Since changing the entire 20-bp resulted in an 8-fold decrease in activity but did not decrease activity to the background level, we hypothesized that there might be another region important for activity. A closer look at upstream region of P_E revealed an inverted repeat sequence, one half of which overlapped the 20-bp (-51 to -70). I made 4 different sets of mutations in the upstream region of P_E (Fig. 2.4A). The activity was decreased when each inverted repeat half-site was mutated, and it decreased to the background level when inverted repeats were disrupted. Mutation of the -33 region resulted in 10-fold decreased activity, as expected if this region is part of the promoter (Fig. 2.4B). These results suggest that RteC may recognize and bind two inverted repeat half-sites, and that the -33 region is also important for full activity. In vitro binding of RteC to this region, as indicated by the ability to shift the

DNA, confirmed this result and added further support to the hypothesis that RteC is a DNA binding protein. The partially purified RteC contained non-specific binding proteins that were observed in gel shift assays. Therefore, I decided to do mutagenesis analysis instead of in vitro footprint assays.

In summary, I have confirmed that RteC is a DNA binding protein and we identified important amino acid residues that might be involved in DNA binding. A possible DNA target sequence on the DNA was also localized.

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Figures and Tables

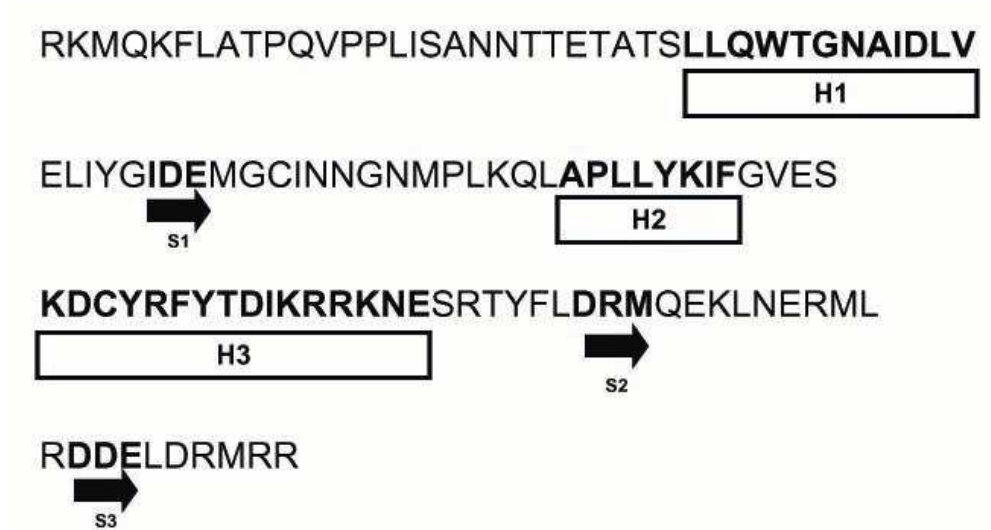


Figure 2.1 The predicted RteC DNA binding domain

The putative RteC C-terminal domain, residues 111 to 217, was deduced based on Robetta prediction results. Based on the Robetta prediction, the putative C-terminal domain might contain a winged helix motif because the human transcriptional factors, E2F-4 and DP2, the closest structural homologs, have winged helix motifs in this region. Amino acid sequences from 111 to 217 were aligned with the DNA binding domains of these structural homologs of RteC, and the putative secondary structure of RteC DNA binding domain was obtained. Three alpha helices are represented as H1, H2, and H3 in boxes. Beta-strands are shown as arrows labeled s1, s2, and s3.

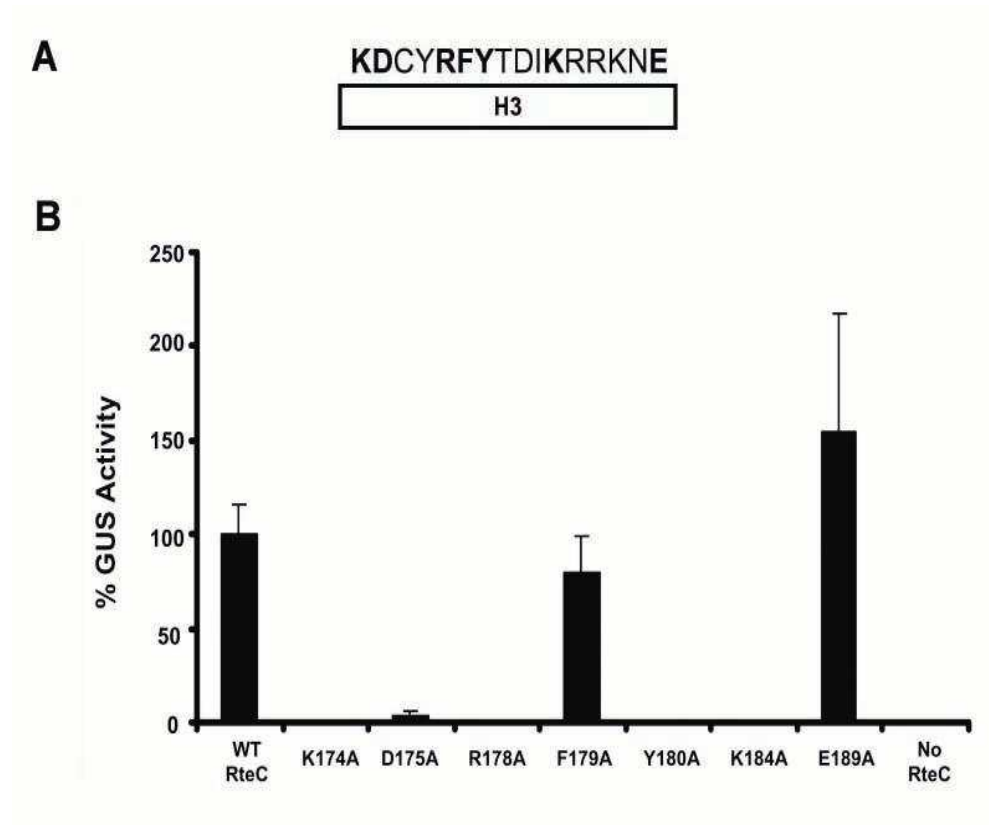


Figure 2.2 Alanine substitutions within the putative RteC helix 3

(A) The amino acid sequence of the predicted helix 3 that was subject to alanine substitutions. Based on sequence alignment with the structural homologs of RteC, fully-conserved, strongly-conserved, and non-conserved amino acid residues (in bold) in the predicted helix 3 were chosen. Amino acid residues that were changed to alanine are in bold. (B) The RteC alanine substitution mutants were placed under control of the heterologous *tetQ* promoter and cloned into the *E.coli-Bacteroides* shuttle vector, pAFD1. The *xis2c::uidA* fusion was provided on a compatible *E.coli-Bacteroides* shuttle vector, pC-COW. In panel B, the values obtained with wild-type RteC were set to 100% and the activity of the each RteC mutant is represented as a percentage of the wild-type P_E value. Without RteC, the GUS activity was less than 1 U/mg protein. The experiments were done in triplicate, and the mean values are shown above. Standard deviations are shown as error bars.

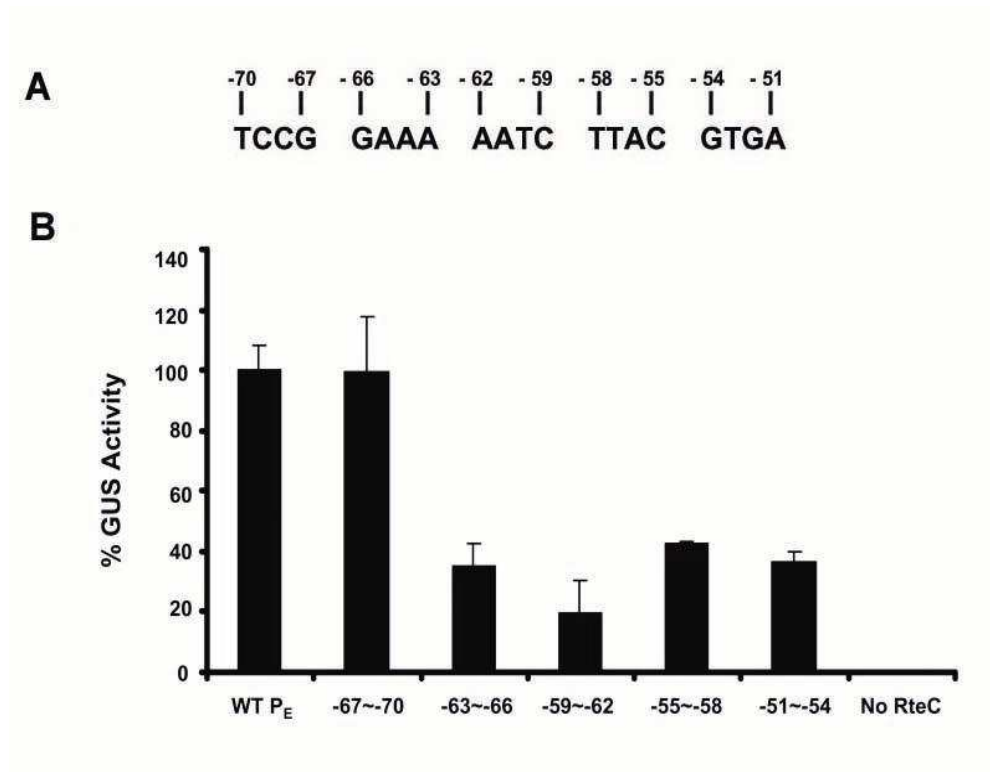


Figure 2.3 Site-directed mutagenesis of upstream region of P_E

(A) In the region upstream of P_E, 5 sets of 4-bp mutations were created in this region by site-directed mutagenesis. (B) In vivo GUS assay results for the mutated constructs are shown. In each case, wild-type P_E or the P_E mutations were fused to the *E.coli uidA* gene creating a translational fusion, and then cloned into the *E.coli-Bacteroides* shuttle vector. His₆-tagged RteC, under control of the heterologous *tetQ* promoter, was cloned into the other *E.coli-Bacteroides* shuttle vector, and then the two plasmids were mobilized into *Bacteroides* strain BT4001. In each panel, the values obtained with wild-type P_E were set to 100% and the activity of the each P_E mutant is represented as a percentage of the wild-type P_E value. Without RteC, the GUS activity was less than 1U/mg protein. The experiments were done in triplicate, and the mean values are shown above. Standard deviations are shown as error bars.

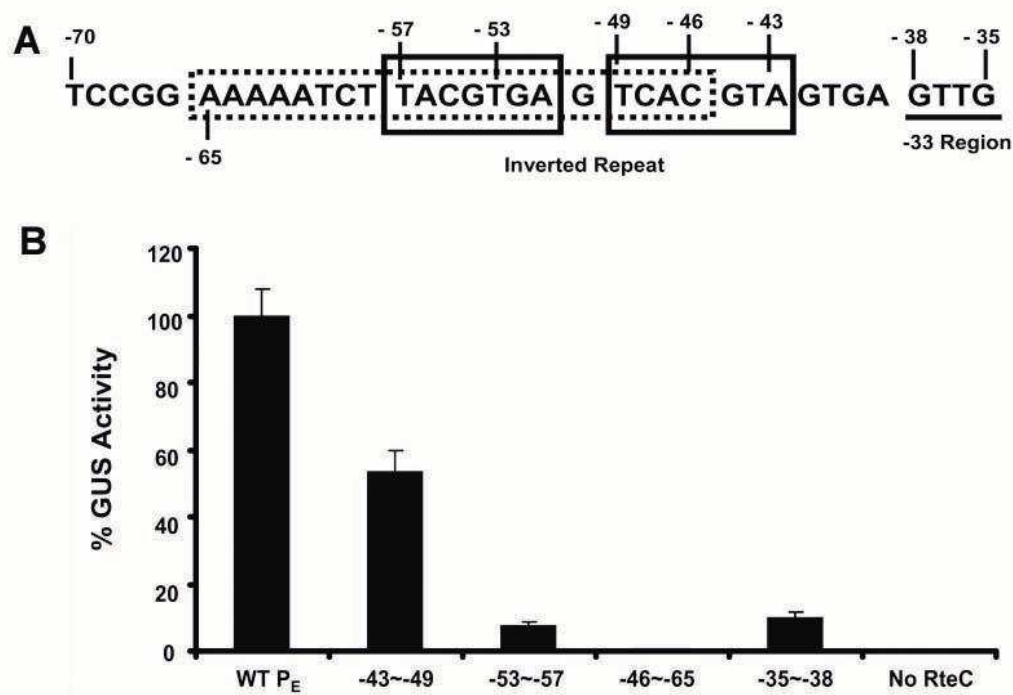


Figure 2.4 Site-directed mutagenesis of the upstream region of P_E

(A) The region between -35 and -65 relative to the *xis2c* transcriptional start site, which contained the inverted half-sites and the -33 promoter region was subject to change. The inverted half-sites are indicated by solid line boxes. The dashed line box shows the 20-bp region initially targeted. -33 promoter region is underlined. 4 different mutated P_E regions were created by site-directed mutagenesis. Changes in -46 to -65 and -35 to -38 were to complementary sequences. The other two P_E mutations, from -43 to -49 and from -53 to -57, were transition mutations to eliminate the inverted repeat sequence. (B) In vivo GUS assay results are shown. In each case, wild-type P_E or the P_E mutations were fused to the *E.coli uidA* gene creating translational fusions, and then cloned into the *E.coli-Bacteroides* shuttle vector. His₆-tagged RteC under control of the heterologous *tetQ* promoter was cloned into the other *E.coli-Bacteroides* shuttle vector, and then the two plasmids were mobilized into *Bacteroides* strain BT4001. In each panel, the values obtained with wild-type P_E were set to 100% and the activity of the each P_E mutant is represented as a percentage of the wild-type P_E value. Without RteC, the GUS activity was less than 1 U/mg protein. The experiments were done in triplicate, and the mean values are shown. Standard deviations are shown as error bars.

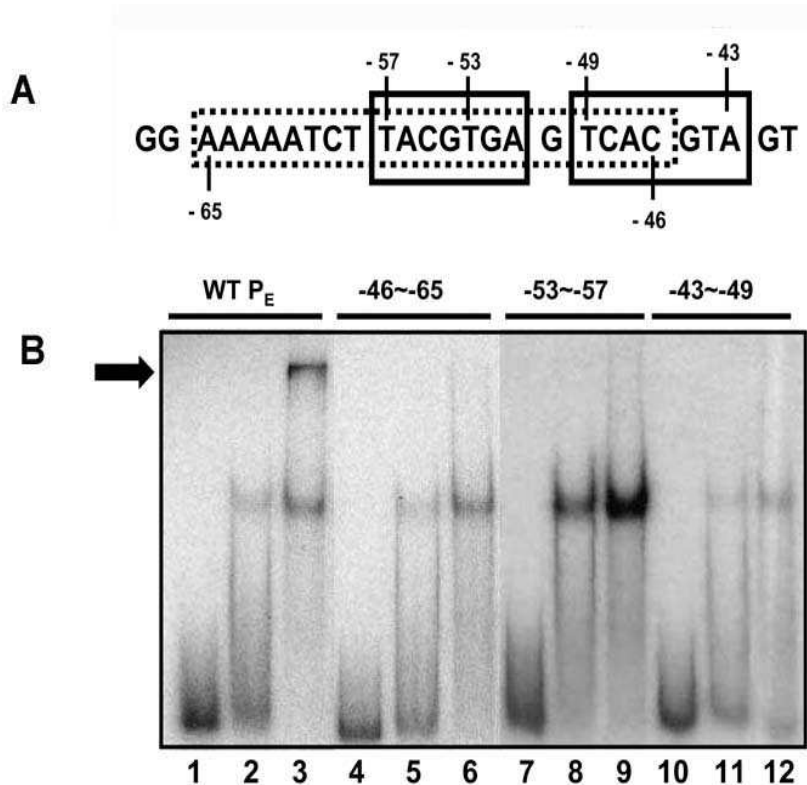


Figure 2.5 Effects of P_E mutation on RteC binding

(A) Sequence of 3 different regions of P_E are shown in boxes. The inverted half-sites are indicated by solid line boxes. The dashed line box shows the 20-bp region initially targeted. Sequences from -43 to -49 and from -53 to -57 relative to the *xis2c* transcriptional start site were changed to disrupt each inverted repeat half-site. The mutations were transition mutations. Sequences from -46 to -65 were changed to the complementary sequence to disrupt the inverted repeat sequence. (B) Gel shift analysis was done to test binding of RteC to wild-type and mutated upstream region of P_E . Reaction conditions were as described in the Materials and Methods section. Lanes 1, 4, 7, and 10, labeled DNA without any protein; lanes 2, 5, 8, and 11, binding mix containing 5 μ g of partially purified empty vector (pET28); lanes 3, 6, 9, and 12, binding mix containing 10 μ g of partially purified RteC. The super shifted protein-DNA complex is indicated by the arrow. The other shifted band comes from an unknown component of the partially purified RteC preparation. The gel is a representative of three independent experiments.

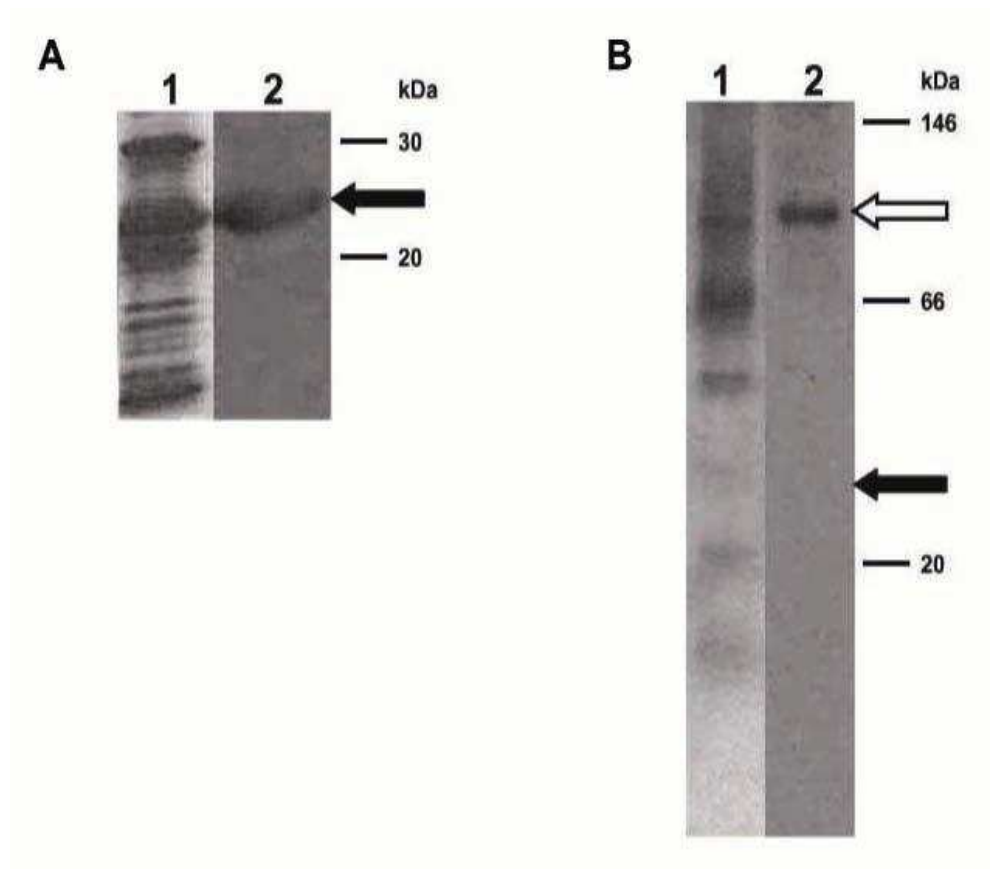


Figure 2.6 Electrophoretic analysis of RteC

(A) Electrophoresis under denaturing conditions. Lane 1, partially purified RteC on SDS gel; lane 2, Western blot analysis of RteC with anti-His antibody. RteC monomer (26 kDa) is indicated by the filled arrow. (B) Electrophoresis under native condition. Lane 1, partially purified RteC on native gel; lane 2, Western blot of RteC with anti-His antibody. Location of RteC monomer (26 kDa) is indicated by the filled arrow. Hollow arrow indicates RteC tetramer (104 kDa).

Table 2.1 Bacterial Strains and Plasmids

Strain or plasmid	Relavant Phenotype	Description and Reference
<i>E.coli</i>		
DH5 α MCR	RecA	Gibco BRL
HB101(RP1)	RecA ^{Str^r}	HB101 containing IncP α plasmid RP1 (88)
BL21-Codon Plus (DE3)RIL	Cam ^r	Cells enable high-level expression of heterologous proteins in <i>E.coli</i> (Stratagene)
<i>B. thetaiotaomicron</i> 5482A		
BT4001	Rif ^r	Spontaneous rifampin-resistant mutant
BT4007	Rif ^r Tc ^r Em ^r	<i>B. thetaiotaomicron</i> 4001 that contains wild-type CTnDOT
Plasmids		
pMJF2	Ap ^r (Em ^r)	A cloning vector to create a uidA fusion, also an <i>E.coli-Bacteroides</i> shuttle vector
pAFD1	Ap ^r (Em ^r)	An <i>E.coli-Bacteroides</i> shuttle vector containing <i>ermF</i>
pC-COW	Ap ^r Tc ^r Cm ^r (Cm ^r)	An <i>E.coli-Bacteroides</i> shuttle vector with IS4351 -cat and <i>Bacteroides</i> plasmid pB8-51 that is compatible with pAFD1 and pMJF2-based vectors (40)
pCR-Blunt	Kn ^r	A 3.5-kb cloning vector for PCR product (Invitrogen)
pET28b	Kn ^r	A plamid for the overexpression of His ₆ -tagged proteins in <i>E.coli</i> (Novagen)
pGFK24.1	Ap ^r	0.3-kb PCR product containing upstream region of the <i>xis2c</i> operon cloned into pCR2.1 (K. Moon, unpublished)
pGFK43.11	Ap ^r (Cm ^r)	A plasmid containing uidA fused to 300 bp upstream region of <i>xis2c</i> start codon cloned into pCR2.1 (K. Moon, unpublished)
pGFK70.1	Ap ^r	1.2-kb PCR product containing RteC _{His6} cloned into PCR2.1 (K. Moon, unpublished)

Table 2.1 (continued)

pGFK76.5	Ap ^r (Cef ^r)	A plasmid containing an in-frame fusion of His ₆ -tagged <i>rteC</i> to the <i>tetQ</i> promoter (K. Moon, unpublished)
pJPARK5	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 4-bp mutated sequence between –67 and –70 upstream region of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK7	Ap ^r (Em ^r)	1.4-kb <i>SphI-SstI</i> fragment from pGFK76.5 containing wild-type pP _Q - <i>rteC</i> ;His ₆ -tagged cloned into <i>SphI-SstI</i> site of pAFD1 (this study)
pJPARK18	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 4-bp mutated sequence between –63 and –66 upstream region of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK19	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 4-bp mutated sequence between –59 and –62 upstream region of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK20	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 4-bp mutated sequence between –55 and –58 upstream region of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK21	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 4-bp mutated sequence between –51 and –54 upstream region of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK22	Ap ^r	0.3-kb PCR product containing 20-bp mutated sequence between –46 and –65 upstream region of the <i>xis2c</i> transcriptional start site cloned into pCR2.1 (this study)

Table 2.1 (continued)

pJPARK26	Kn ^r	760-bp PCR product containing wild-type <i>rteC</i> cloned into pCR-Blunt (this study)
pJPARK28	Ap ^r	0.3-kb PCR product containing 7-bp mutated sequence between –43 and –49 upstream region of the <i>xis2c</i> transcriptional start site cloned into pCR2.1 (this study)
pJPARK32	Kn ^r	760-bp <i>NdeI-XhoI</i> fragment from pJPARK26 cloned into <i>NdeI-XhoI</i> site of pET28b creating His ₆ -tagged <i>rteC</i> (this study)
pJPARK37	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 7-bp mutated sequence between –43 and –49 upstream of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK38	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 20-bp mutated sequence between –46 and –65 upstream of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK39	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 4-bp mutated sequence between –35 and –38 upstream of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)
pJPARK49	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (K174A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)
pJPARK50	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (R178A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)
pJPARK51	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (Y180A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)

Table 2.1 (continued)

pJPARK63	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (D175A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)
pJPARK64	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (E189A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)
pJPARK65	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (K184A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)
pJPARK66	Ap ^r (Em ^r)	1.2-kb PCR product containing RteC _{His6} mutant (F179A) cloned into <i>NcoI-SmaI</i> site of pC-COW (this study)
pJPARK76	Ap ^r	0.3-kb PCR product containing 5-bp mutated sequence between –53 and –57 upstream of the <i>xis2c</i> transcriptional start site cloned into pCR2.1 (this study)
pJPARK78	Ap ^r Tc ^r Cm ^r (Cm ^r)	<i>uidA</i> fused 0.3-kb PCR product containing 5-bp mutated sequence between –53 and –57 upstream of the <i>xis2c</i> transcriptional start site cloned into the <i>NruI-SphI</i> site of pC-COW (this study)

Chapter 3

EXCISION PROTEINS ARE POSITIVE REGULATORS OF *BACTEROIDES* CTNDOT TRANSFER

3.1 Introduction

In *Bacteroides* species, conjugative transposons (CTns) play an important role in spreading antibiotic genes (8, 9). CTnDOT is a 65-kb CTn containing a tetracycline resistance gene, *tetQ*, and an erythromycin resistance gene, *ermF* (1, 10, 11). A distinctive feature of the CTnDOT family of CTns is that both excision and transfer are stimulated by exposure of donor cells to tetracycline (17, 18). In the presence of tetracycline transcriptional attenuation regulates production of proteins encoded a three-gene operon, which include the resistance protein TetQ and a two-component regulatory system, RteA and RteB (16, 17). Both RteA and RteB were identified as regulatory proteins based on amino acid similarity to other known two-component regulatory systems, with RteA being the histidine kinase-environmental sensor and RteB being the transcriptional activator (12, 13). RteA and RteB activate the expression of a third regulatory gene, *rteC*, which controls the expression of an operon containing genes involved in excision (6, 13). In the case of

CTnDOT excision, 2 genes (*xis2c* and *xis2d*) are required for excision, and a third gene (*exc*) is stimulatory (2, 14). This effect has been shown both in vivo and in vitro. These three genes, along with the CTnDOT integrase (IntDOT), coordinate the excision of CTnDOT from its site in the host chromosome (2).

A recent surprising finding was that the CTnDOT excision proteins were also necessary for transfer gene regulation (5, 18). The expression of the transfer gene operon by tetracycline required not only the presence of the regulatory genes (*rteA*, *rteB*, and *rteC*) but also the excision genes (*xis2c*, *xis2d*, and *exc*) (5). When the excision genes were placed under control of a heterologous promoter, expression of the transfer genes occurred in the absence of *rteA*, *rteB*, and *rteC* (5). Although the excision genes were clearly required for transfer gene expression and had been shown to be required individually in in vitro excision assays, the role of Xis2c and Xis2d on transfer gene regulation had not been established. There was some evidence that deletion of *exc* abolished production of the transfer proteins, as indicated by the Western blot analysis (18). It was not clear whether Exc was required to stimulate *tra* gene expression or was stimulatory as in the case of excision. Gel shift assays using a 450-bp, sequence that contained 80-bp of the region upstream of the first gene of the transfer operon (*traA*), showed that several

overlapping DNA fragments within this region were bound by Xis2c and Xis2d, but no shift (or binding) was detected with Exc (J. Lee, unpublished). We showed the footprint of the Xis2d binding to a 50-bp region immediately upstream and adjacent to the promoter site. We also determined the effects of mutations within this region on the in vivo transcription and the in vitro gel shift assays. Our results support the hypothesis that both Xis2c and Xis2d are DNA binding proteins, that Xis2d is required for transfer gene regulation and that Xis2c makes a contribution but has a less dramatic effect. Exc appears to bind cooperatively with Xis2d.

3.2 Materials and methods

Strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1.

The concentrations of the antibiotics used were: ampicillin , 100 µg/ml; chloramphenicol (Cm), 15 µg/ml; erythromycin (Em), 10 µg/ml; gentamicin (Gen), 200 µg/ml; rifampicin (Rif), 10 µg/ml, tetracycline , 1 µg/ml. All the *Escherichia coli* strains were grown in Lysogeny Broth (LB) or on LB agar at 37°C and the *Bacteroides* strains were grown in either Trypticase Yeast Extract Glucose (TYG)

medium or Supplemented Brain Heart Infusion (BHIS) medium at 37°C under anaerobic conditions.

Transfer of shuttle vectors

Vectors were transferred from *E.coli* donors to *Bacteroides* recipients by triparental matings using nitrocellulose filters on BHIS agar plates incubated aerobically as previously described (17). The *Bacteroides thetaiotaomicron* recipients contained either the entire CTn, CTnERL which is very closely related to CTnDOT or a strain, BT4001ΩQABC that contained *tetQ-rteA-rteB* and *rteC* integrated in the chromosome containing the *traA::uidA* reporter vector, pGW40.5 (5) depending on the experiment being performed.

Construction of deletion mutants

Deletions in *xis2c*, *xis2d*, and *exc* are shown in Fig. 3.1. 7.6-kb *SphI-SstI* fragment of pKSO1 (3) containing the excision gene operon was cloned into the *SphI-SstI* site of pAFD1 to create pJPARK82. This plasmid was used as a wild-type to compare effects of deletion mutations on activity. 2.1-kb *SphI-SstI* fragment of pGW41(3) containing *xis2c* and *xis2d*, but no *exc*, was cloned into the *SphI-SstI* site

of pAFD1 (pJPARK83). This plasmid was used as an *exc* deletion mutant. For in-frame deletion of *xis2c*, a 3.7-kb *PstI-SstI* fragment from pGW53 (G. Whittle, unpublished) was cloned into the *PstI-SstI* site of pUC19, creating pGRW52. pGW53 is a pGERM-T vector containing P_E -*xis2c-xis2d-exc*. Then a 650-bp *PstI-HincII* fragment from pGRW52 was taken out, and 316-bp *PstI-HincII* PCR product containing P_E -part of *xis2c* (31 out of 119 a.a.) was cloned into the *PstI-HincII* site of pGRW52 to make pGRW53 Δ 2c'. To make an in-frame deletion of *xis2d*, the 1.1-kb *BstEII-PstI* fragment from pGW53 was removed, and a 665-bp *BstEII-PstI* fragment containing P_E -*xis2c* PCR product was cloned into the *BstEII-PstI* site of pGW53, creating pGRW53 Δ 2d'. The *PstI-SstI* fragments containing each in-frame deletion were cloned into the *E.coli-Bacteroides* shuttle vector, pAFD1, to create pGRW53 Δ 2c (in-frame deletion of 264 nucleotides from 5' end) and pGRW53 Δ 2d (in-frame deletion of the entire *xis2d*). Deletion mutations were confirmed by sequence analysis (UIUC DNA Core facility, Urbana, IL).

Site-directed mutagenesis

To test the effects of various mutations in the region identified by the footprint analysis of Xis2d binding, several 5~7 bp site-directed mutations were

created in the upstream region of TraA. The effects of these mutations were tested in vivo by GUS fusion analysis and in vitro by gel shift analysis. Site-directed mutagenesis was done using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and PfuUltra DNA Polymerase (Stratagene). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). pJPARK85. A pCR-Blunt vector (Invitrogen), containing the 450-bp region upstream of the TraA (-138 ~ +352), was used as DNA template in site-directed mutagenesis of the upstream region of the transfer operon promoter (P_{traA}). The PCR amplicons containing different mutations were transformed into *E.coli* DH5 α MCR competent cells (Bethesda Research Laboratories Inc., Gaithersburg, MD), and cells were grown in Lysogeny Broth (LB) agar medium containing kanamycin (50 μ g/ml) overnight. Plasmids were isolated, and then sequenced to confirm the mutation (ACGT Inc. Wheeling, IL). Once mutations were confirmed, the *SphI-SmaI* restriction fragments were isolated and ligated into the *SphI-SmaI* site of and *E.coli-Bacteroides* shuttle vector, pMJF2, to create an *traA::uidA* translational fusion. Mutated forms of the *traA* upstream region were affected in vitro by gel shift analysis (see later section).

GUS assay

The *uidA* reporter gene on pMJF2 encodes an *E.coli* β -glucuronidase (GUS). GUS assays were done by the procedure of Feldhaus et al (4). One unit was defined as 0.01A₄₁₅(absorbance at 415 nm) per minute at 37°C. Protein concentrations were determined by the method of Lowry et al (7) .

Overexpression and purification of Xis2d

pCMK1042, a pET28 vector (Novagen), containing native *xis2d* (C. Keeton, unpublished) was used to overexpress Xis2d. *Escherichia coli* BL21 (DE3) *ihfA* was used as the host strain. Cells were grown overnight at 37°C in 10ml Lysogeny Broth (LB) medium with chloramphenicol (50 µg/ml) and kanamycin (30 µg/ml). The overnight cultures were used to inoculate 500 ml of LB medium containing chloramphenicol (50 µg/ml) and kanamycin (30 µg/ml). The culture was incubated with vigorous shaking at 30°C. Cells were grown until O.D.₆₀₀ was 0.6, and isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were grown at 25°C for 4 hours with vigorous shaking. Cells were sonicated and cell extract was prepared and purified by FPLC heparin column.

Xis2d was eluted from heparin column using high salt buffer (50 mM Na-P; pH 7.0, 2 M NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT). Active fractions were collected and dialyzed in dialysis buffer (5 mM Na-P; pH 7.0, 1 M NaCl, 1 mM EDTA, 40% glycerol, 1 mM DTT) and stored at -80°C until use (C. Keeton, unpublished).

Overexpression and purification of His₆-tagged Exc was done as documented by Sutanto et al (15).

Overexpression of Xis2c

Although Xis2d could be overexpressed and partially purified, attempts to purify Xis2c was unsuccessful. Accordingly, for in vitro analysis, we used a partially purified preparation of Xis2c for analyzing the binding of Xis2c to mutant forms of the *traA* upstream region. pCMK936, a pET30 vector (Novagen), containing native *xis2c*, (C. Keeton, unpublished) was used to overexpress Xis2c. *Escherichia coli* BL21 (DE3) *ihfA* was used as the host strain. Cells were grown overnight at 37°C in 10 ml Lysogeny Broth (LB) medium with chloramphenicol (50 µg/ml) and kanamycin (30 µg/ml). The overnight cultures were used to inoculate 500ml of LB medium containing chloramphenicol (50 µg/ml) and kanamycin (30 µg/ml). The culture was incubated with vigorous shaking at 37°C. Cells were grown

until O.D.₆₀₀ was 0.3~0.4, and isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were grown at 25°C for overnight with vigorous shaking. Cells were harvested by centrifugation at 7,000 rpm for 20 min at 4°C. The supernatant fluid was discarded, and the cells were stored at –80°C until use. The frozen cell pellet was resuspended in 20ml low salt buffer (50 mM Na-P; pH7.0, 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM DTT) to which two Complete EDTA-free protease inhibitor tablets (Roche Applied Science) and 20 mg of lysozyme had been added. The cells were lysed by sonication (10 sec sonication and 59.9 sec on ice, 10 times). Cell lysate was separated by centrifugation at 12,000 rpm for 60 min at 4°C. The crude cell extract was dialyzed overnight in a Slide-A-Lyze cassette (Thermo Scientific) against dialysis buffer (50 mM Na-P; pH7.0, 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM DTT, 20% glycerol), and stored at –80°C until use. Attempts were made to purify Xis2c by heparin agarose column, but purification of Xis2c was not successful (C. Keeton, unpublished).

Gel shift assays

A 450-bp DNA fragment containing region from –138 to +352 relative to *traA* transcriptional start site were utilized in the gel shift assay experiments. This

fragment was used to ensure that all binding sites were included, and the same fragment was also used for *traA::uidA* fusions. DNA fragments were prepared from plasmids containing wild-type or mutated P_{traA} region by restriction enzyme digest (*SphI* and *SmaI*) followed by gel extraction. DNA fragments were then labeled with gamma-[³²P] ATP using PCR, and purified. The DNA fragment (approximately 5 ng) and either Xis2c crude extract, partially purified Xis2d or Exc were mixed in 10 µl binding buffer and be incubated for 15 min at 25°C. The 5X binding buffer contains 250 mM Tris-HCl (pH 8.0), 5 mM EDTA, 250 mM NaCl, 50% Glycerol, and 3.6 µg/ml herring sperm DNA. The binding mixture was loaded onto a 6% DNA Retardation Gel (Invitrogen), and 0.5X Tris-borate-EDTA buffer (pH 8.0) was used as the running buffer. After electrophoresis, the gels were dried and subjected to autoradiography.

DNaseI footprint assays

The upstream region of *traA* was amplified by PCR using fluorescently labeled primers, with either top or bottom strand primers labeled with 6-carboxyfluorescein (6-FAM). The PCR product contained region from -134 to +78. The bp numbers are relative to *traA* transcriptional start site (+1). Individual

footprint reactions were initiated by mixing footprint binding buffer (0.5 mM CaCl₂, 2.5 mM MgCl₂, 10 mM Tris-HCl; pH7.5), 6-FAM labeled DNA (10 pmol), and water to a final volume of 18 µl. Binding reactions were performed by adding 2µl of various amount of Xis2d (60~240 ng) followed by 30 min incubation at 25°C. 2 µl of DNaseI (New England Biolabs) at an approximately 1:2000 dilution in 1X DNaseI buffer (0.5 mM CaCl₂, 2.5 mM MgCl₂, 10 mM Tris-HCl; pH7.5) was added to each reaction, and incubated for 2 min at 25°C. The digestion reactions were stopped by adding 40 µl of 0.5 M EDTA (pH 8.0). DNA segments were then immediately purified using QIAGEN PCR cleanup kit. Fragment analysis was done at the UIUC DNA Core Facility (Urbana, IL), and the Genemapper software (Applied Biosystems) was used for further analysis.

3.3 Results

Contribution of Xis2c, Xis2d, and Exc to the regulation of CTnDOT transfer gene expression

Previous work (18) showed that Exc helped to enhance the conjugative transfer of pLYL72, a plasmid containing the transfer operon of CTnDOT that are necessary for transfer (18). Western blot analysis showed that deleting part of *exc* abolished the enhanced production of three transfer proteins, TraG, TraN, and TraP

(18). It is still not clear whether Exc is required or stimulatory because the Western blot analysis was not very sensitive. The effect of in-frame deletion in Xis2c or Xis2d on *tra* gene regulation had not been previously tested. A vector containing *xis2c* and *xis2d* but no *exc* resulted in the loss of the increased transfer of pLYL72 and in the loss of the increased synthesis of key transfer proteins (e.g. TraG) (18). To test whether Xis2c, Xis2d, and/or Exc are required to regulate expression of the transfer gene operon, we made in-frame deletions in *xis2c* and *xis2d*. The downstream sequences of *xis2c* and *xis2d* were deleted for the exc mutant (Fig. 3.1A). Both wild-type and deletion mutants were cloned into shuttle vectors then tested in strain BT4001ΩQABC (pGW40.5) for their abilities to induce the transcription of the *traA::uidA* fusion when the cells were grown in the presence of tetracycline (Fig. 3.1B). A strain containing the *tetQ-rteA-rteB*, and *rteC* region inserted in the chromosome (18) was used because the deletion clones were under the control of the natural excision operon promoter (P_E). When the wild-type excision operon containing all three genes on a plasmid (pJPARK82) was introduced into the strain, the GUS activity of the *traA::uidA* fusion was increased 150-fold in the presence of tetracycline compared to the background level (Fig. 3.1B). When the vector contained a deletion in *xis2c* (pGRW53Δ2c), the GUS activity of the cells

induced by tetracycline was reduced 58% relative to the wild-type ($\Delta xis2c$ in Fig. 3.1B). Thus, the deletion in *xis2c* reduced but did not abolish *tra* gene expression. The tetracycline-induced activity of *traA::uidA* was decreased 97% when pGRW53 Δ 2d was present ($\Delta xis2d$ in Fig. 1B), indicating that Xis2d is more important than Xis2c for the transcriptional control of the transfer operon. Exc deletion resulted in only 16% decreased activity (Δexc , Fig. 3.1B).

Xis2d specifically binds upstream region of *traA* operon

Gel shift assay results showed that both Xis2c and Xis2d were able to shift a DNA fragment containing a 450-bp upstream region of TraA and the promoter P_{traA}. No gel shift was seen if an unrelated DNA was used, so the binding was specific (J. Lee, unpublished data). Xis2d could be partially purified, but in the case of Xis2c we had to use cell extracts due to loss of activity of the protein when it is further purified. Thus, we were limited to using Xis2d in footprint experiments to try to localize the region(s) where the Xis proteins were binding. Preliminary gel shift assay results showed that three different DNA fragments within this fragment (-89~+251, -164~+15, -89~+15) were bound by Xis2d (J. Lee, unpublished) (Fig. 3.2A, upper diagram). We used a 212-bp fragment that contained the region of these

three different DNA fragments to identify possible binding sites for Xis2d (Fig 3.2A, lower diagram). DNaseI footprint assays were performed using fluorescently labeled DNA probes containing the 212-bp region from –134 to +78 relative to the *traA* transcriptional start site with the 6-carboxyfluoresceine (6-FAM) label on the 5' end of either the top or bottom strands. The elution profile of Xis2d binding to upstream region of *traA* (Fig. 3.2B) showed a reproducible depression of peak height within the region from –81 to –30 relative to the *traA* transcriptional start site (Fig. 3.2A indicated by shaded box, Fig. 3.2C). The base pairs that were protected from the DNaseI digest indicated by the arrows (Fig. 3.2C) are bases that are bound by Xis2d and thus are presumed to be important.

The effect of P_{traA} region mutations on binding of Xis2d to DNA

To determine if the Xis2d binding sites identified by DNaseI footprint assays were important, gel shift assays were done. In the gel shift assays, the 450-bp fragments containing either wild-type or site-directed mutations that are indicated by the boxed regions in Fig 3.3A were used. The arrows again indicate the bases protected by Xis2d (Fig. 3.3A). A distinct shift of the fragment was observed when both wild-type Xis2d and wild-type fragment were present. (Fig. 3.3B, lane 2,

indicated by a filled arrow). Five different mutations were made by site-directed mutagenesis within the 50-bp footprint region of Xis2d, -81 to -30 relative to the *traA* transcriptional start site (Figure 3.3A). Each region subjected for site-directed mutagenesis was changed to its complementary sequence to conserve the melting temperature characteristics. When regions outside of the Xis2d binding site were mutated (-81~-77 and -39~-34, Fig. 3.3A), the DNA fragment was still shifted (Fig. 3.3B, lanes 4 and 12). Mutations within region that might contain Xis2d binding site resulted in loss of binding (Fig. 3.3B, lanes 6, 8, and 10).

The effect of P_{traA} region mutations on binding of Xis2c to DNA

Although mutations within footprinted region had little effect on Xis2d binding, it was possible that Xis2c binding might be affected. Gel shift assays were done as described for Xis2d above. Due to technical difficulties concerning loss of activity observed here and in vitro excision assays (14), we were forced to use Xis2c crude cell extracts as our source of Xis2c. Empty vector crude cell extract (same vector but no *xis2c*) was used as a negative control. With wild-type Xis2c and wild-type P_{traA} , the shift was observed (Fig. 3.4B, lane 2, indicated by solid arrow). When the binding mixture contained only empty vector crude extract, the two bottom

bands were present (Fig. 3.4B, indicated by hollow arrows). We checked whether mutations within 50-bp footprint region with Xis2d would affect the ability of Xis2c to bind to the DNA. All 5 of the mutations prevented the shift of the fragment. This result confirmed that Xis2c is a DNA binding protein, and that the region from –81 to –30 is more important for the observed binding of Xis2c than for Xis2d.

The effect of mutations on in vivo transcriptional activity

With the varied results observed for the in vitro binding of Xis2d and Xis2c on the 50-bp region, we also wanted to test whether these mutations in the region from –81 to –30 had any effects on the in vivo transcriptional activity. The same mutations tested in the in vitro gel shift assays were cloned into pMJF2 to make *traA::uidA* fusions. These plasmids were then mobilized into BT4004 where an intact CTnERL was used to provide the tetracycline regulated genes (*tetQ-rteA-rteB* and *rteC*) as well as the entire excision operon. Since the expression of excision genes is regulated by tetracycline, we again compared the GUS activity with and without tetracycline. The results of the five different mutations within the region upstream of Xis2d footprint region on the GUS activity of *traA::uidA* fusions are shown in Figure 3.5B. With the wild-type sequence, the GUS activity was increased

50-fold in the presence of tetracycline. The GUS activity was reduced nearly to background by most of the mutations. Clearly, although the in vitro effects of the mutations on Xis2d binding were minimal, the effects of the mutations in vivo were significant. Either the in vitro assay did not accurately reflect the effects of mutations in this region or Xis2d alone is not sufficient for the effects it has in vivo.

Exc binds the upstream region of *traA* cooperatively with Xis2d

A possible explanation of the lack of effect in vitro of *traA* upstream mutations on Xis2d binding was that in vivo Xis2d is interacting with proteins such as Exc. In the gel shift assays, Exc did not shift DNA containing the upstream region of the *traA* operon (Figure 3.6A), however it is present on the clones which were used to determine the in vivo contribution of Xis2c and Xis2d to the transcription of *traA*. Xis2c plus Exc, without Xis2d, had less than 5% the transcriptional induction as Xis2d plus Exc. To test the possibility that there is some measurable interaction of Exc with Xis2d, we checked whether Exc could bind this region cooperatively with Xis2d. The results of the gel shift assays are shown in Figure 3.6. 30 ng of Xis2d was used in these assays. With this concentration of Xis2d protein, only the half of DNA was shifted (Fig. 3.6B, lane 2, indicated by filled arrow). Addition of

increasing amounts of Exc was added which resulted in complete shifting of the DNA fragment and the appearance of a super-shift band (Fig. 3.6B, lanes 3 to 8, indicated by a hollow arrow). At 64 ng of Exc, all of the fragment is either in the first Xis2d band or in the super shift band. From 128 ng of Exc or higher, the fragment migrated to the wells (Fig. 3.6A). Thus Exc appears to interact cooperatively with Xis2d in some way, possibly by facilitating Xis2d binding to DNA (Fig.3.6B).

3.4 Discussion

It is unusual that CTnDOT excision genes (*xis2c*, *xis2d*, and *exc*) are involved in both excision and transfer. Previously, *xis2c* and *xis2d* were designated as *orf2c* and *orf2d*, but these genes were re-named since they play an important role in excision. Xis2c contains a helix-turn-helix (HTH) motif, and both Xis2c and Xis2d are small basic proteins. However, they do not have a primary sequence homology to known proteins in the databases (14). Exc was shown to have topoisomerase function in vitro, although this activity appears not to play a role in the activity of Exc in vivo (14). Therefore, my hypothesis was that Xis2c and Xis2d are DNA binding proteins that bind the upstream region of the transfer operon as

transcriptional activators, and Exc might work cooperatively with these proteins to bind to upstream region of *traA* promoter (P_{traA}) and enhance the expression of the transfer genes. This suggests that the binding of Exc either to Xis2d or to facilitate Xis2d binding to DNA is involved rather than some effect of Exc on DNA super-coiling in the region.

Deletion of Xis2c showed 58% reduced activity, but not to the background level (Fig. 3.1B). This might be because Xis2d and Exc were still present in the absence of Xis2c. Xis2d and Exc bind cooperatively to DNA (Fig. 3.6B) and this might explain the 42% residual activity. It was not determined whether Xis2d alone contributed entire 42% activity in the absence of Xis2c. Xis2d deletion resulted in 97% decreased activity (Fig. 3.1B). With Exc deletion, the activity was decreased 16%, suggesting that Xis2c and Xis2d together could make near wild-type transcription. Without excision genes, the activity was less than 0.1% of wild-type activity in the presence and absence of tetracycline.

The preliminary gel shift assay used 3 different overlapping DNA fragments that all contained a common region between -164 and +251 relative to the *traA* transcriptional start site. Since all fragments were shifted with Xis2c and Xis2d, I decided to focus on the common region to footprint of Xis2c, Xis2d, and Exc.

Neither Xis2c nor Exc had a specific binding region that could be protected from DNaseI digest. I had to use crude cell extract of Xis2c due to technical difficulties to purify active Xis2c, and non-specific binding proteins present in the cell extract might have interfered specific binding of Xis2c to the *traA* upstream region. Also, Xis2c did not overproduce very well, so the concentration of Xis2c could be too low. Partially purified Exc alone did not shift DNA and did not give a footprint within the upstream region of *traA* (Fig. 3.6A), however, Exc interacts cooperatively with Xis2d to enhance binding of Xis2d to the upstream region of *traA* in vitro. This supports the hypothesis that Exc interacts with Xis2d in the positive regulation of transfer operon.

I made 5 different 5~7 bp mutations within the *traA* upstream. Region footprinted by Xis2d to test the effects of these mutations on in vivo activity and in vitro DNA binding. The footprint region was 50-bp but there were no repeats or inverted repeats observed. Originally, I planned to make 6 different mutations within the region footprinted by Xis2d (-81 ~ -30), but I was unsuccessful in constructing mutations between -49 and -56. The in vivo GUS assays showed the decreased activity to the background level with most of the mutations, and the same mutations eliminated Xis2c binding. Mutations within Xis2d footprinted site resulted in loss of

Xis2d binding (Fig. 3.3B), while two mutations (-81~-77 and -39~-34) did not affect binding of Xis2d to DNA (Fig. 3.3B).

In summary, a 50-bp region upstream of *traA* was footprinted by Xis2d and this region was also shown to be important for both Xis2c and Xis2d binding by in vitro gel shift assays. The footprinted region between -81 and -30 relative to the *traA* transcriptional start site was located immediately upstream of the promoter of transfer operon. This supports our hypothesis that Xis2c and Xis2d are DNA binding proteins that activate transfer operon expression. Mutagenesis of region upstream of *traA* showed that this region was critical for in vivo activity of the *traA::uidA* fusion and both Xis2c and Xis2d binding in vitro. Previous work showed that Exc is required for enhancement of expression of downstream transfer genes in the presence of tetracycline (18). In vivo GUS assays with Exc deletion mutant, however, showed 16% decreased activity. Exc might be involved in translational regulation of genes downstream of *traA*. By performing in vitro gel shift assays with Xis2d and Exc, I presented the first evidence that Exc works cooperatively with Xis2d to enhance binding to DNA and positively regulates transfer operon expression. Exc might also work cooperatively with Xis2c but further purification of Xis2c is needed to do test this.

Acknowledgements

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Figures and Tables

Figure 3.1

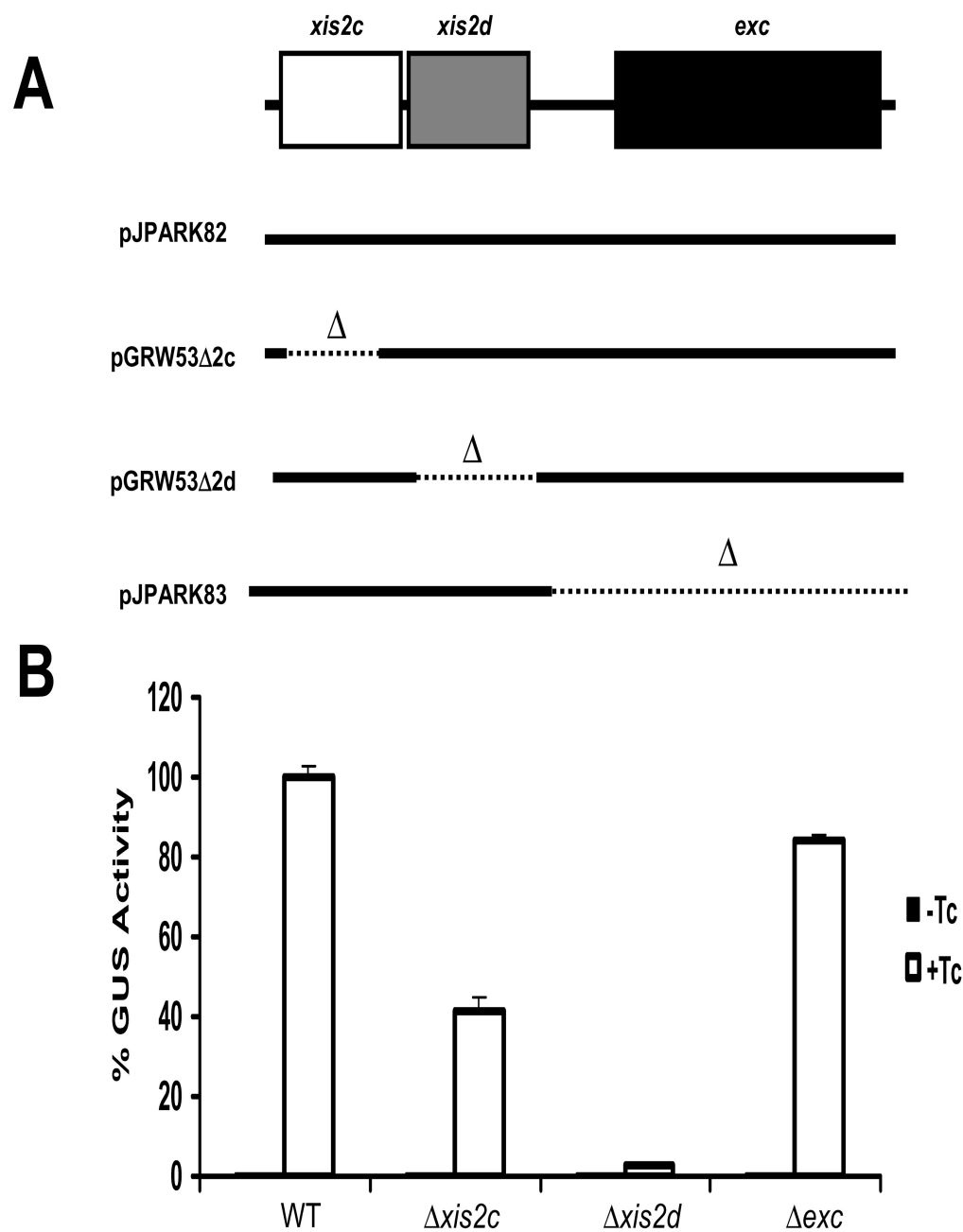


Figure 3.1 (continued)

Figure 3.1 Effect of deleting *xis2c*, *xis2d*, and *exc* on the regulation of the CTnDOT transfer operon

(A) Wild-type excision genes, *xis2c* or *xis2d* in-frame deletion, and *exc* deletion mutants (pJPARK82, pGRW53Δ2c, pGRW53Δ2d, and pJPARK83, respectively) constructed to test whether Xis2c, Xis2d, and Exc are required to regulate transfer gene expression. (B) Plasmid containing either wild-type genes or a deletion mutant of *xis2c*, *xis2d*, or *exc* was mobilized into BT4001ΩQABC containing the *traA::uidA* fusion (pGW40.5). The values obtained with wild-type excision operon and tetracycline induction were set to 100% and the activity with the each deletion mutant is represented as a percentage of the wild-type value. The experiments were done in triplicate, and the mean values are shown above. Standard deviations are shown as error bars.

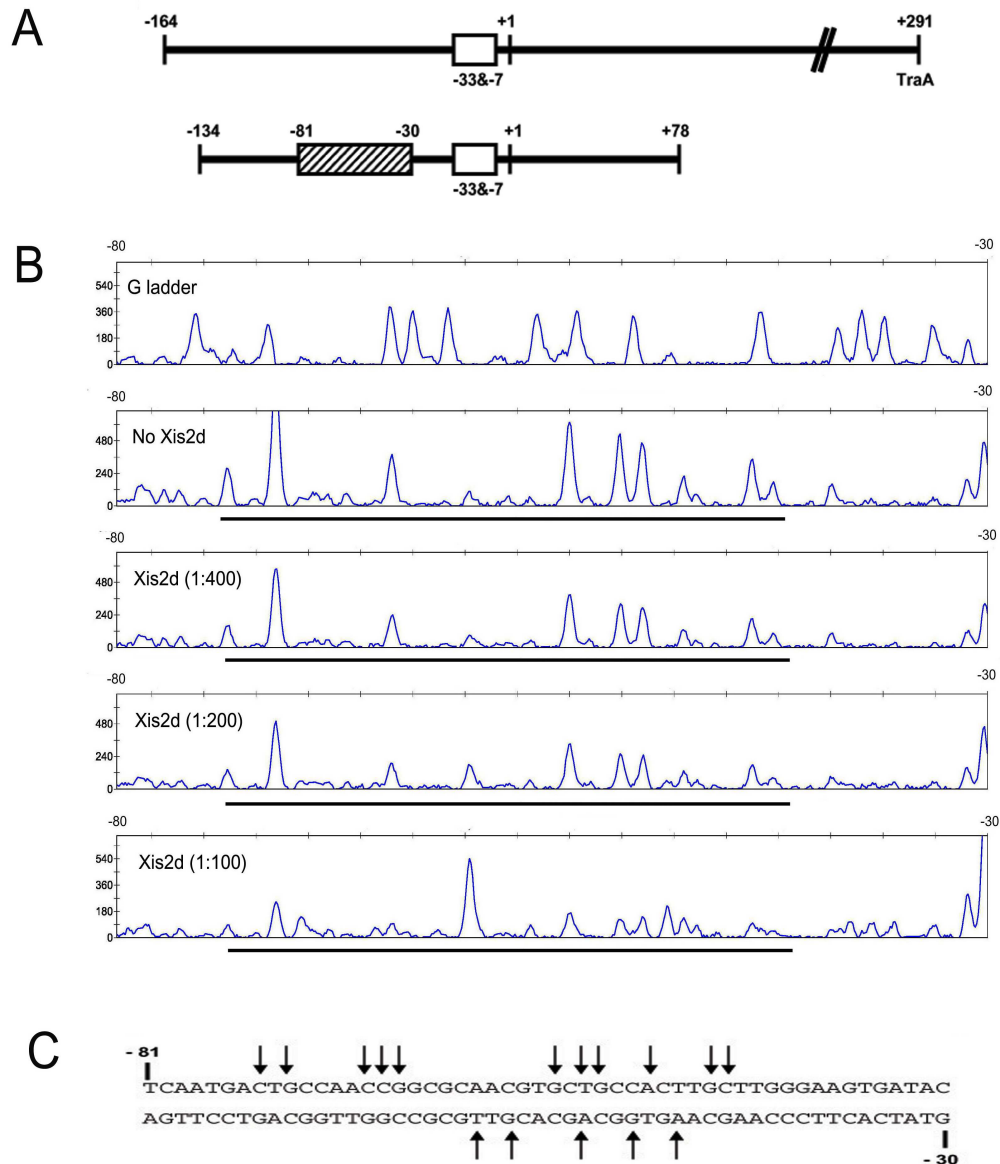


Figure 3.2 DNaseI footprint analysis of Xis2d to the *traA* promoter region

(A) DNA fragments used to determine the location of the Xis2d binding site within upstream region of the *traA* promoter are shown. The numbers above the diagram indicate the position relative to the TIS (+1) of the *traA* gene. The putative *tra* promoter (-33&-7) is indicated with white box. Shaded box includes the region protected from DNaseI digest. (B) FAM-labeled DNA fragments were sequenced by running parallel didioxy G, C, T, and A reactions. Here, G sequencing ladder is

Figure 3.2 (continued)

shown as an example. DNaseI protection sites are underlined. (C) Sequence of upstream region of *traA* is shown. Base pairs that were protected from DNaseI digest are indicated by vertical arrows.

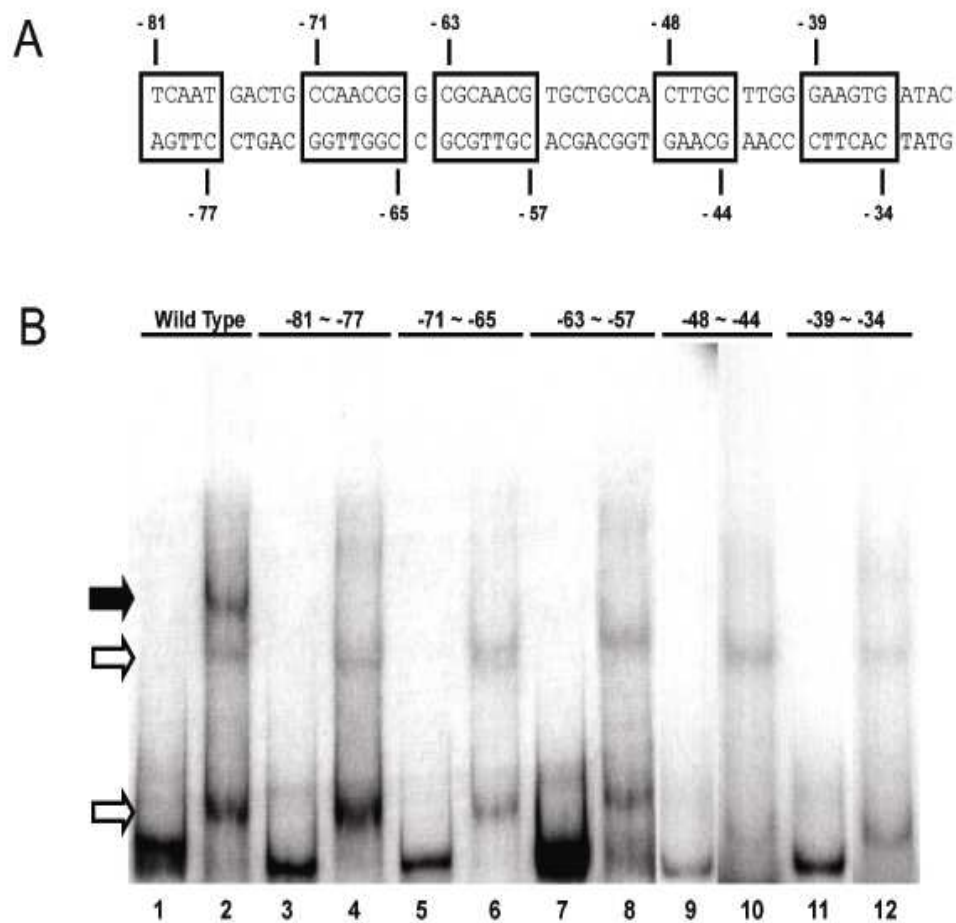


Figure 3.4 Effects of P_{traA} mutations on Xis2c binding

(A) Sequence of 5 different regions of P_{traA} that were subjected for site-directed mutagenesis are shown in boxes. Sequences from -81 to -77, -71 to -65, -63 to -57, -48 to -44, and -39 to -34 relative to the *traA* transcriptional start site were changed to its complementary sequences. (B) Gel shift analysis was done to test binding of Xis2c to wild-type and mutated upstream region of P_{traA} . Reaction conditions were as described in the Materials and Methods section. Lanes 1, 3, 5, 7, 9, and 11; labeled DNA without any protein; lanes 2, 4, 6, 8, 10, and 12; binding mix containing 9 μ g of Xis2c crude cell extract. The other shifted bands indicated by hollow arrows come from an unknown component of the crude cell extract preparation. The super-shifted protein-DNA complex is indicated by the arrow. The gel is a representative of three independent experiments.

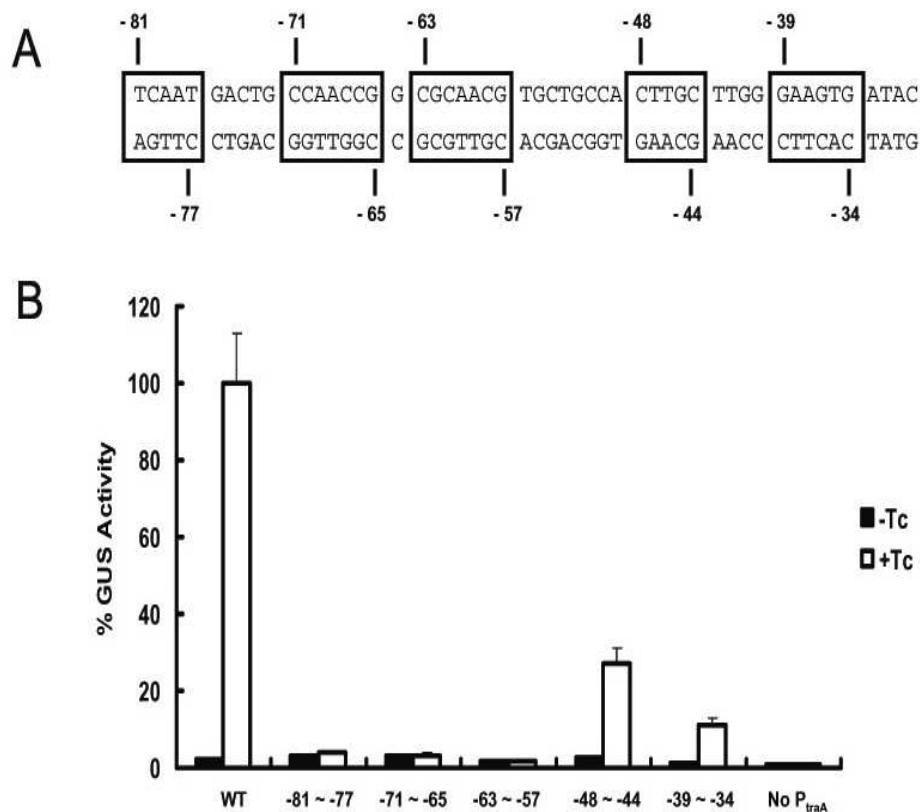


Figure 3.5 Site-directed mutagenesis of the upstream region of P_{traA}

(A) Sequence of 5 regions of P_{traA} that were subjected for site-directed mutagenesis are shown in boxes. sequences from -81 to -77, -71 to -65, -63 to -57, -48 to -44, and -39 to -34 relative to the *traA* transcriptional start site were changed to its complementary sequences. (B) In vivo GUS assay results are shown. In each case, wild-type P_{traA} or the P_{traA} mutations were fused to the *E.coli uidA* gene creating translational fusions, and then mobilized into *Bacteroides* strain BT4004. In each panel, the values obtained with wild-type P_{traA} induced by tetracycline; +Tc) were set to 100% and the activity of the each P_{traA} mutant is represented as a percentage of the wild-type P_{traA} value. Without P_{traA} , the GUS activity was less than 1U/mg protein. The experiments were done in triplicate, and the mean values are shown. Standard deviations are shown as error bars.

Figure 3.6 (continued)

upstream P_{traA} region. Filled arrow indicates the DNA shift by Xis2d. The super-shifted protein-DNA complex is indicated by hollow arrow. The gel is a representative of three independent experiments.

Table 3.1 Bacterial Strains and Plasmids

Strain or plasmid	Relavant Phenotype	Description and Reference
<i>E.coli</i>		
DH5 α MCR	RecA	Gibco BRL
HB101(RP1)	RecA Str^r	HB101 containing IncP α plasmid RP1 (88)
BL21(DE3)	Cam r	Cells enable high-level expression of heterologous proteins in <i>E.coli</i> (Stratagene)
<i>B. thetaiotaomicron</i> 5482A		
BT4001	Rif r	Spontaneous rifampin-resistant mutant
BT4001 Ω QABC	Tc r	BT4001 with site-specific insertion of <i>tetQ</i> , <i>rteA</i> , <i>rteB</i> , and <i>rteC</i> into the chromosome via NBU1 mini-insertion vector
BT4004	Rif r Tc r	<i>B. thetaiotaomicron</i> 4001 that contains wild-type CTnERL
Plasmids		
pMJF2	Ap r (Em r)	A cloning vector to create a <i>uidA</i> fusion, also an <i>E.coli</i> - <i>Bacteroides</i> shuttle vector
pC-COW	Ap r Cm r (Cm r)	pC-COW is pTC-COW digested by <i>PvuII</i> and relegated
pGW40.5	Ap r Cm r (Cm r)	Contains fusion <i>P_{traA}::uidA</i> cut out of pMJF2 by <i>PvuII</i> and cloned into NruI site of pC-COW, along with wild-type <i>traA</i> promoter (5)
pCR-Blunt	Kn r	A 3.5-kb cloing vector for PCR product (Invitrogen)
pET27b	Kn r	A plasmid for the overexpression of proteins in <i>E.coli</i> (Novagen)
pET30	Kn r	A plasmid for the overexpression of proteins in <i>E.coli</i> (Novagen)
pJPARK82	Ap r (Em r)	7.6-kb <i>SphI</i> - <i>SstI</i> fragment from pKSO1(18) containing the excision gene operon cloned into the <i>SphI</i> - <i>SstI</i> site of pAFD1 (this study)

Table 3.1 (continued)

pJPARK83	Ap ^r (Em ^r)	2.1-kb <i>SphI-SstI</i> fragment from pGW41(18) containing <i>xis2c</i> , <i>xis2d</i> , but <i>exc</i> cloned into the <i>SphI-SstI</i> site of pAFD1 (this study)
pJPARK85	Kn ^r	0.5-kb <i>SphI-SmaI</i> fragment containing -138 to +352 relative to the <i>traA</i> transcriptional start site cloned into pCR-Blunt (This study)
pJPARK90	Kn ^r	0.5-kb <i>SphI-SmaI</i> fragment containing 5-bp mutation cloned into pCR-Blunt 5-bp (-81 to -78 relative to the <i>traA</i> transcriptional start site) was changed to complementary sequence by site-directed mutagenesis (This study)
pJPARK95	Kn ^r	0.5-kb <i>SphI-SmaI</i> fragment containing 7-bp mutation cloned into pCR-Blunt 5-bp (-63 to -57 relative to the <i>traA</i> transcriptional start site) was changed to complementary sequence by site-directed mutagenesis (This study)
pJPARK100	Ap ^r (Em ^r)	0.5-kb <i>SphI-SmaI</i> fragment from pJPARK85 cloned into the <i>SphI-SmaI</i> site of pMJF2 (This study)
pJPARK104	Ap ^r (Em ^r)	0.5-kb <i>SphI-SmaI</i> fragment from pJPARK90 cloned into the <i>SphI-SmaI</i> site of pMJF2 (This study)
pJPARK108	Ap ^r (Em ^r)	0.5-kb <i>SphI-SmaI</i> fragment from pJPARK95 cloned into the <i>SphI-SmaI</i> site of pMJF2 (This study)
pJPARK119	Kn ^r	0.5-kb <i>SphI-SmaI</i> fragment containing 7-bp mutation cloned into pCR-Blunt 5-bp (-71 to -65 relative to the <i>traA</i> transcriptional start site) was changed to complementary sequence by site-directed mutagenesis (This study)

Table 3.1 (continued)

pJPARK120	Kn ^r	0.5-kb SphI-SmaI fragment containing 7-bp mutation cloned into pCR-Blunt 5-bp (-48 to -44 relative to the traA transcriptional start site) was changed to complementary sequence by site-directed mutagenesis (This study)
pJPARK121	Kn ^r	0.5-kb SphI-SmaI fragment containing 6-bp mutation cloned into pCR-Blunt 5-bp (-39 to -34 relative to the traA transcriptional start site) was changed to complementary sequence by site-directed mutagenesis (This study)
pJPARK122	Ap ^r (Em ^r)	0.5-kb <i>SphI-SmaI</i> fragment from pJPARK119 cloned into the <i>SphI-SmaI</i> site of pMJF2 (This study)
pJPARK123	Ap ^r (Em ^r)	0.5-kb <i>SphI-SmaI</i> fragment from pJPARK120 cloned into the <i>SphI-SmaI</i> site of pMJF2 (This study)
pJPARK124	Ap ^r (Em ^r)	0.5-kb <i>SphI-SmaI</i> fragment from pJPARK121 cloned into the <i>SphI-SmaI</i> site of pMJF2 (This study)
pGRW53Δ2c	Ap ^r (Em ^r)	3.5-kb fragment containing in-frame deletion of <i>xis2c</i> cloned into pAFD1 (This study)
pGRW53Δ2d	Ap ^r (Em ^r)	3.5-kb fragment containing in-frame deletion of <i>xis2d</i> cloned into pAFD1 (This study)
pCMK936	Kn ^r	365 bp <i>NdeI-HindIII</i> fragment containing native <i>xis2c</i> cloned into the <i>NdeI-HindIII</i> site of pET30a (C. Keeton, unpublished)
pCMK1042	Knr	458 bp <i>NdeI-XhoI</i> fragment containing native <i>xis2d</i> cloned into the <i>NdeI-XhoI</i> site of pET27b (C. Keeton, unpublished)

Chapter 4

CONCLUDING REMARKS AND FUTURE STUDIES

Conjugative transposons (CTn) play an important role in spreading antibiotic resistance genes among bacteria by horizontal gene transfer (1, 3, 4, 8, 13). CTnDOT is a CTn found in *Bacteroides* species (11, 12), and responsible for spreading antibiotic resistance genes among *Bacteroides* species (10, 12, 16). Previous work in our laboratory has shown that regulation of CTnDOT is a very complex process, and that regulation of CTnDOT excision and transfer is triggered by tetracycline (2, 7, 15).

At first, I studied a positive regulator of CTnDOT excision, RteC. Little was known about this regulatory protein when I started to work on this project. I found that RteC is a winged-helix type DNA binding protein that binds two inverted repeat half sites within the upstream region of the excision gene operon, and activates the expression of the excision genes. Because of the lack of primary sequence homology of RteC to known protein databases, I looked for the structural homologs using the Robetta protein structure prediction server. Using the three-dimensional structural model prediction, I was able to find homologs of this unique

protein, RteC. It is also interesting to note that a couple of human transcriptional factors, E2F-4 and DP2, are the most likely structural homologs of RteC.

Once excision of CTnDOT occurs, the next step is transfer from donor to recipient by conjugation. For my second project, I focused on the positive regulators of CTnDOT transfer. In CTnDOT, three excision proteins (Xis2c, Xis2d, and Exc) are required for both excision and transfer (2, 6, 14, 15). My work confirmed that both Xis2c and Xis2d are DNA binding proteins that bind to the upstream region of the transfer operon to activate the transfer genes. Exc did not bind to DNA by itself, but it appears to work cooperatively with Xis2d to enhance Xis2d binding to DNA.

I have completed the initial characterization of the positive regulators of CTnDOT excision and transfer. My hypothesis is that RteC forms a tetramer to bind two inverted repeat half-sites within a region upstream of the promoter of the excision operon, P_E , and that Xis2c and Xis2d interact with each other to bind to the upstream region of the CTnDOT transfer operon to activate transfer gene expression. The next step is to take a more in-depth look at the protein complexes these proteins form.

4.1 Further characterization of RteC

I used His₆-tagged RteC for in vitro gel shift assays to increase the ease of purification and detection. Yet, there were other non-specific binding proteins present after cobalt column purification, and this resulted in a non-specific DNA-protein complex in gel shift assays. Therefore, I used partially purified empty vector as a negative control. To get past this hurdle, further purification of RteC will need to be done. Affinity purification using the His₆-tag will be done followed by either heparin column purification or size exclusion chromatography. Size exclusion chromatography followed by native gel analysis and Western blotting will also be done to confirm RteC oligomerization result shown in Chapter 2. Once pure RteC has been obtained, the next step will be the crystallization of RteC and RteC-DNA. This experiment will confirm that RteC has a winged helix motif and that the two inverted repeat half-sites are contacted by the putative helix 3.

My work showed that RteC is a DNA binding protein that binds two inverted repeat half-sites within the upstream region of the excision operon. Future work with DNaseI footprint assays will be done with further purified RteC to confirm both the in vivo GUS assays and the in vitro gel shift assay results. Further, the upstream region of *xis2c* can be amplified by PCR using fluorescently labeled

primers, with either the top or bottom strand primers labeled with 6-carboxyfluorescein (6-FAM). Different concentrations of RteC will be added to binding mixtures containing 6-FAM labeled DNA probe followed by DNaseI digest. Footprint assay samples will be immediately cleaned up by Qiagen PCR clean up kit, and subjected to the fragment analysis. Lastly, results will be analyzed using GeneMapper software to interpret the footprint data.

4.2 Mutagenesis of Xis2c and Xis2d

Xis2c and Xis2d play an important role in both excision and transfer of CTnDOT. Yet, it was not determined in detail how these proteins mediate excision and activation of transfer gene expression. Accordingly, Xis2c and Xis2d mutants that are defective in excision could be selected and tested for their abilities to activate transfer gene expression. Due to the lack of primary sequence homology of known databases proteins, random mutagenesis will be done to identify important amino acid residues within Xis2c and Xis2d for excision. Hydroxylamine, a chemical that targets G:C pairing and deaminates the cytosine to uracil, will be used to generate random Xis2c and Xis2d mutants.

To isolate mutants defective for excision, a blue/white colony selection in *E.coli* will be done. This screen has already been developed in the Gardner laboratory. There are five components required for excision of CTnDOT: Xis2c, Xis2d, IntDOT, BHFa, and the *attL/attR* sites (14). In an *E.coli* in vivo system, two plasmids will be used to select excision mutants. An indicator plasmid that contains *intDOT* and *lacZ* cloned between the *attL/attR* sites. During excision, Xis2c and Xis2d bind *attL/attR* sites (14). This plasmid also contains $\lambda attP$ site, which allows it to integrate into the *E.coli* chromosome via site-specific recombination with the host encoded *attB* site. The second plasmid will contain *xis2c* and *xis2d* and be provided in trans. If Xis2c and Xis2d mutants are able to bind at *attL/attR* site, they will disrupt the expression of *lacZ* resulting in a white colony. Therefore, blue colonies will be selected as excision mutants.

Once excision mutants are selected, these mutants will be cloned into *E.coli-Bacteroides* shuttle vector, pAFD1, and mobilized into the *Bacteroides* strain BT4001 Ω QABC containing pGW40.5. BT4001 Ω QABC contains the central regulatory region (*tetQ-rteA-rteB*, and *rteC*) on the chromosome, and pGW40.5 contains a *traA::uidA* fusion. If excision mutants are functional in activation of transfer gene expression, they will bind the upstream region of *traA* provided by

pGW40.5, and show high GUS activity in the presence of tetracycline. To confirm the in vivo GUS assay results, in vitro gel shift assays will be done with excision mutants to test whether these mutants are still capable of binding the transfer region.

4.3 Determination of protein-protein interactions between Xis2c, Xis2d and Exc

Xis2c and Xis2d are small basic DNA-binding proteins that are located right next to each other. Therefore, it is possible that these proteins form oligomers that bind to the transfer region. To test for protein-protein interactions, co-expression of Xis2c and Xis2d, followed by size exclusion chromatography will be done. In this case, one of the proteins will be labeled with ³⁵S-methionine for detection. Then the labeled protein will be separated on a two dimensional SDS-PAGE gel and subjected to autoradiography for quantification and identification.

Another way to detect protein-protein interaction is protein complex immunoprecipitation (Co-IP). Co-IP works by selecting an antibody that targets a known protein in the hypothesized protein complex (5, 9), in our case, antibodies will be made for Xis2c, Xis2d and Exc. By targeting a known protein with an antibody it may possible to pull an entire protein complex out of solution and thereby isolate the other proteins that form the complex. It will be interesting to see

whether all three antibodies identify the same complex. This experiment could give us some insights into the protein composition and stoichiometry of the active complex.

4.4 DNaseI footprint assays of P_{traA} with Xis2c

I showed that Xis2c is a DNA binding protein that binds the upstream region of P_{traA}. To identify the region Xis2c binds and to confirm in vitro gel shift assay results in Chapter 3, DNaseI footprint assays of P_{traA} with Xis2c will be done. To do this, further purification of Xis2c will be done. Purification of Xis2c using heparin column resulted in loss of activity. Therefore, cell extract containing overexpressed Xis2c will be purified using size exclusion chromatography followed by ion-exchange chromatography. Once pure Xis2c is obtained, crystallization of protein and protein-DNA will also be done to determine the structure and regions that contact DNA.

4.5 Determine tetracycline regulation of mobilization genes

In CTnDOT, mobilization genes are located upstream region of transfer genes. By performing qualitative RT-PCR, I and another student in our laboratory (J.

Waters) found that *mob* genes are regulated by tetracycline. Quantitative RT-PCR will be done to confirm this result. I showed in Chapter 3 that a 50-bp region upstream of *traA* is important for Xis2c and Xis2d binding. Since the promoter regions of both *mob* and *tra* genes are adjacent to each other, it is possible that the two excision proteins, Xis2c and Xis2d, that were shown to bind upstream of the *traA* promoter also bind the *mob* promoter and enhance the expression of both mobilization and transfer genes in the presence of tetracycline. Therefore, I checked whether upstream region of *mob* genes contain the similar region to 50-bp upstream of *traA* that were found to be important for Xis2c and Xis2d. Although no homology was found, Gel shift assays will be done to check whether Xis2c and Xis2d could bind P_{mobA} region. If these proteins bind region upstream of *mobA*, then DNaseI footprint assays will be done to identify their binding sites and these will be compared to sites obtained of Xis2d.

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Curriculum Vitae

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EDUCATION

Ph.D. Microbiology

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RESEARCH EXPERIENCE

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Characterization of *Bacteroides* CTnDOT regulatory protein, RteC

Characterization of *Bacteroides* CTnDOT excision proteins

(Xis2c, Xis2d, and Exc) as positive regulators of transfer genes

Missouri University of Science and Technology (2003~2005)

Advisor: Dr. David Westenberg

Iron, heme, and oxygen regulation of the *hemA* and *sdh* genes from the soybean symbiont *Bradyrhizobium japonicum*

TECHNICAL SKILLS

DNA

Preparation of chromosomal and plasmid DNA : *Bacteroides* and *E.coli*

Bacterial conjugation between *Bacteroides* and *E.coli*

Primer design

DNA amplification by PCR

Gene cloning

Site-directed mutagenesis

Gel electrophoresis

Transformation

RNA

Preparation of total RNA: *Bacteroides*

Qualitative RT-PCR

Protein

Protein overexpression and coexpression

Protein purification: affinity column and FPLC

Oligomerization

Western blot

Protein assays

Reporter gene assays

Gel shift assays: both radioactive and non-radioactive

Footprint assays: non-radioactive

Etc.

Preparation of anaerobic media for *Bacteroides*

Bioinformatics: accessing, analyzing, and interpreting using NCBI databases

Using Robetta structure prediction server to find structural homologs

Sequence alignment using ClustalW

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PUBLICATIONS

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PRESENTATIONS

Park and Salyers, Midwest Microbial Pathogenesis Conference, St. Louis, MO, 2010 (Poster Presentation)

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TEACHING EXPERIENCE

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